



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/82, 5/10, A01H 5/00 // C12N 15/54, 15/12, 15/55 C12N 15/29	A1	(11) International Publication Number: WO 90/08830 (43) International Publication Date: 9 August 1990 (09.08.90)
(21) International Application Number: PCT/GB90/00110 (22) International Filing Date: 26 January 1990 (26.01.90) (30) Priority data: 8901677.8 26 January 1989 (26.01.89) GB (71) Applicant: IMPERIAL CHEMICAL INDUSTRIES PLC [GB/GB]; Imperial Chemical House, Millbank, London SW1P 3JF (GB). (72) Inventors: BRIDGES, Ian, George ; Box 30A, R.R.I, Sla- ter, IA 50244 (US). BRIGHT, Simon, William, Jonathan ; 24 Pound Lane, Marlow, Bucks SL7 2AY (GB). GREENLAND, Andrew, James ; "The Cabin", Raymill Road East, Maidenhead, Berkshire SL6 8SX (GB). SCHUCH, Wolfgang, Walter ; 14 Greenfinch Close, Heathlake Park, Crowthorne, Berkshire RG11 6TZ (GB).		(74) Agent: HUSKISSON, Frank, Mackie; Imperial Chemical Industries plc, Legal Department-Patents, P.O. Box No. 6, Bessemer Road, Welwyn Garden City, Herts AL7 1HD (GB). (81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (Eu- ropean patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI pa- tent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: HYBRID SEED PRODUCTION (57) Abstract <p>Male sterility is imparted to a plant by a cascade of gene sequences which expresses a protein which disrupts the biosynthesis of viable pollen. Expression of the disrupter protein is restricted to male parts of the plant by an upstream promoter sequence which is specific to male flowers, the male specific promoter being under control of an operator sequence. The cascade also includes a gene encoding a repressor protein specific for that operator. Expression of the repressor protein is under control of a chemically inducible promoter which is inducible by the application to the plant by, spraying or like process, of an exogenous chemical. In the absence of the exogenous chemical inducer, no repressor protein is expressed, resulting in expression of the disrupter protein and, consequently, male sterility. Fertility may be restored to the plant, when required for maintenance of the line, by spraying with the inducer, resulting in expression of the repressor which binds the operator and inhibits expression of the disrupter protein.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

HYBRID SEED PRODUCTION

This invention relates to a method for the production of hybrid plants. More particularly, the invention relates to the molecular control of fertility in crop plants.

5 Agriculture uses many crop plants for the production of food for human consumption, for commercial processes yielding products for human consumption, for animal feedstuff production, for the development of industrial products and other
10 purposes. The process invariably involves the planting by the farmer of seed which has been purchased from a seed producer. The product produced by the crop be it the whole plant, the seed or fruit of the plant, is harvested and is
15 then used for the various food applications mentioned above. In addition to purchasing seed from a seed company, the farmer also may plant back some seed saved from a previous year's crop. This, however, is only economically useful for crops
20 which are used as inbreds or outcrossed seed. It is not economically advantageous for hybrid crops, which are planted to realise the increase in productivity achieved by heterosis. The major crops planted in the major agricultural regions are
25 planted as hybrid crops, which guarantee the farmer considerable yield increases.

Production of hybrid seed by specialised seed companies is a costly and complicated process. The process involves the breeding and selection of inbred parental lines, which in suitable combinations give progeny which exhibit maximal adaptation to environmental conditions with realisation of maximal yield. For hybrid seed production inbreds are chosen as either male or female parent in a cross yielding F1 hybrid seed. As most crops are hermaphroditic, the female parent in a cross has to be emasculated in order to avoid self-pollination during seed production. For open pollinating plants like maize a special planting regime is used in order to minimise self-pollinating of the female parent. This involves the separation of those plants to be used as males from the female parents. This allows easy separation of F1 hybrid seed at the end of the season.

Emasculation can be achieved either mechanically as it is used for maize, manually as it is used for tomato, or chemically as it is used for wheat or rice, and genetically using cytoplasmic male sterility (CMS) or genetic incompatibility as it is used for oil seed rape, sugar beet and others. These approaches vary in their complexity of agricultural practices or plant manipulation, but have in common, that they are costly to administer, complex to carry out and relatively inefficient depending on which system is used. This inefficiency arises from the fact that fertile plants need to be sterilised for seed production, which takes place in very large acreages depending on the crop. Therefore, large

scale manipulation of females need to be carried out. Treatments also need to yield seed which is viable in the next generation which the farmer plants.

5 An object of the present invention is to obviate or mitigate the aforesaid disadvantages.

 According to the present invention there is provided a plant gene construct which includes a disrupter protein gene capable of disrupting the
10 biogenesis of viable pollen, and a gene control sequence which includes a promoter sequence inducible by external application of an exogenous chemical inducer to a plant containing the construct.

15 Preferably, the control sequence includes an operator controlling said disrupter protein gene and a repressor gene encoding a repressor protein adapted to bind said disrupter protein gene operator, said repressor protein gene being under
20 control of said chemically inducible promoter.

 Preferably also the construct includes a male flower specific operator sequence operatively linked to said disrupter protein gene, for restricting expression of the disrupter protein
25 gene to male flower parts of a plant.

 The invention also provides transformed plants and plant parts such as cells, protoplasts and seeds incorporating the construct of the invention.

30 In a preferred embodiment of the invention there is provided a recombinant DNA construct for insertion into the genome of a plant to impart restorable male sterility thereto, comprising:
(a) a first gene promoter sequence responsive to

the presence or absence of an exogenous chemical inducer,

(b) a gene encoding a repressor protein under control of the said first promoter sequence;

5 (c) an operator sequence responsive to the said repressor protein;

(d) a second gene promoter sequence expressible only in male parts of a plant; and,

10 (e) a gene encoding a protein inhibitor of a plant characteristic essential to the production of viable pollen;

whereby the presence or absence of the exogenous chemical inducer enables selection of male fertility or sterility.

15 Further according to the invention there is provided a plant which is restorably male sterile, in which said plant contains, stably incorporated in its genome, the recombinant DNA construct defined above.

20 It is preferred that the said first promoter promotes expression of the repressor protein in response to stimulation by the exogenous chemical inducer whereby in the absence of the chemical inducer no repressor protein is expressed to
25 interact with the operator thus permitting expression of the gene encoding the inhibitor of male fertility and in the presence of the chemical inducer repressor protein is expressed thereby preventing expression of the gene encoding the
30 inhibitor of male fertility and restoring the plant to the fertile state. Thus the construct of the invention contains several operatively linked sequences (a) above will be referred to for convenience as "the chemical switch": (b) as "the

repressor sequence" (c) as "the operator" (d) as
"the MFS control" (i.e male flower specific
control) and (e) as the disrupter gene. The
essential elements of each of the sequences and
5 their interaction will be described below with
reference to the accompanying drawings.

This invention enables the production of
inbreds which are rendered male sterile using
various molecular techniques and approaches. These
10 plants require a chemical switch system for the
reversal of fertility (reversible male sterility;
RMS). Both aspects of the system are inherited as
Mendelian characters. This will be achieved
through the insertion into the plant genome of the
15 molecular elements required for the controlled
function of RMS.

The invention can be used for any mono- or
di- cotyledonous plant which the breeder or grower
wants to produce as F1 hybrid seed and for which
20 suitable transformation techniques are or become
available, particularly maize, wheat, sunflower,
oil seed rape, tomato and other vegetables, sugar
beet and ornamental foliage and flowering plants.
It has great advantages in reduction in crop
25 management costs associated with F1 hybrid seed
production, ease of purity control of hybrid seed,
maintenance of RMS lines and populations, transfer
of RMS between lines and populations etc.

In one specific application we shall describe
30 the production of plants, particularly inbred
plants, which are rendered sterile using molecular
engineering approaches. These plants can be
reversed to fertility using a chemical spray which
leads to the restoration of fertility using a

molecular control cascade. The method presented here consists of a number of individual components which are subject to separate patent applications which disclose wider applications of the components.

1. THE OVERALL PROCESS

Figure 1 of the drawings is a block diagram of the DNA construct of the invention in the "male sterile" state. In the absence of the exogenous chemical inducer, the chemical switch is inactive and no repressor protein is expressed by the repressor sequence. In the absence of the repressor protein, the operator sequence permits expression of the disrupter protein in male specific tissue, expression being specifically directed to male parts of the plant by the presence of the MFS control sequence. The outcome being that the plant is male sterile, is unable to produce viable pollen and thus unable to self-pollinate. The practical utility of this effect is that if such plants, representing the female of an intended cross, are planted proximate the intended male parent of the cross, it will be pollinated by pollen from the intended male.

Figure 2 shows the operation of the construct in the "male fertile" state. When the chemical inducer is brought into contact with the plant, the chemical switch is activated causing the repressor protein to be expressed. The repressor protein then binds the operator, inhibiting expression of the disrupter protein and restoring male fertility. The practical utility of this mode of operation of the construct is that it allows production of viable pollen and self-pollination of the plant

containing the construct in order that the line may be perpetuated for future use.

2. THE CHEMICAL SWITCH

5 A large number of plant promoters are assumed to be induced using chemical signals. However, it has only been demonstrated in few examples that the specific chemicals switch on gene expression in the tissue required for this model. The gene of particular interest is the gene encoding the 27k
10 subunit of glutathione-S-transferase II (GSTII). This gene is induced specifically upon treatment of plant tissues, particularly developing anthers using chemical safeners. One such safener is N,N-diallyl-2,2- dichloroacetamide, but there are
15 related compounds which have improved mobility characteristics in plants tissues, combined with improved persistence for this application, efficacy and safety. These compounds have been described in the literature.

20 It is obvious that additional chemically induced promoters can be used in this scenario. Some of these may be of plant origin, others may be of fungal or yeast origin. It is implied in the present application that those promoters and
25 chemical combinations suitable for the RMS procedure can be used in case of GSTII and safeners.

3. THE REPRESSOR AND OPERATOR SEQUENCES

30 In a first embodiment we propose to use the well- characterised interaction between bacterial operators with their repressors to control the expression of the 'killer' gene function. Bacterial repressors, particularly the lac repressor, or repressors used by 434, P22 and lambda

bacteriophages can be used to control the expression in plant cells very effectively.

5 In a second embodiment it is possible to utilise 'pseudo-operators', operators which are similar but not identical to the normally used operators in a particular operator-repressor combination. We have demonstrated that using a suitable selection system mutant repressors can be generated which recognise pseudo-operators found in
10 plants genes. We describe below the selection of mutant repressors recognising pseudo-operators which are found in plant genes.

Another approach for the down regulation of the 'killer' genes which can be considered is the
15 use of antisense RNA. This has been demonstrated to work well for the regulation of polygalacturonase expressed during tomato fruit ripening and is described in the literature.

4. MALE FLOWER SPECIFIC EXPRESSION CASSETTE

20 As already mentioned, the expression of the 'killer' genes has to take place in male flower specific tissues (MFS). These can be tissues found in the developing anthers, in tissues associated with the developing anthers and pollen.

25 DNA promoter sequences which drive the expression of genes in MFS tissues can be achieved using established protocols for the identification of genes expressed in MFS tissues through differential screening of cDNA libraries cloned in
30 various vector systems, the isolation of genes encoding these cDNAs from genomic libraries using bacteriophage lambda vectors, and the characterisation of their promoter sequences using

DNA sequencing and analytical plant transformation experiments.

5. DISRUPTER GENE

Inhibition of pollen formation will be
5 achieved by using novel 'killer' genes which, when
expressed specifically in male flowers during
pollen formation (for details see above), will lead
to cell death of the anthers and associated
tissues, pollen mother cells, pollen and associated
10 tissues. This will lead to the abortion of pollen
formation, and plants which are male sterile. The
origin of the 'killer' genes can be from a variety
of naturally occurring sources, eg human cells,
yeast cells, plant cells, fungal cells, or they can
15 be totally synthetic genes which may be composed of
DNA sequences some of which are found in nature,
some of which are not normally found in nature or a
mixture of both. The 'killer' genes will have
preferably an effect on mitochondrial metabolism,
20 at it has been quite clearly demonstrated that
ample energy supply is an absolute requirement for
the production of fertile pollen. However, it is
also envisaged that the 'killer' function can be
effectively targeted to other essential biochemical
25 functions such as DNA metabolism, protein
synthesis, and other metabolic pathways. Two such
DNA constructs consist of those sequences encoding
the mammalian brown adipose tissue uncoupling
protein or variants thereof, or a synthetic gene
30 which consists of a mitochondrial targeting domain,
and a lipophilic domain which allows insertion of
the protein into the mitochondrial membrane.

6. PRODUCTION OF AN EXPRESSION MODULE CONSISTING OF MFS PROMOTER SEQUENCES AND 'KILLER' GENES

Production of an expression module which consists of the male flower specific gene control sequences (MFS control) and the 'killer' genes will be done using established molecular techniques. The expression of this module in elite inbreds will lead to the production of the 'killer' gene product only in male flower specific tissues. This will lead to the production of male sterile plants.

7. TRANSFORMATION

Transgenic plants are obtained by insertion of the constructs described into the genome of the plants. The specific transformation procedure employed for insertion of the gene constructs of this invention into the plant genome is not particularly germane to this invention. Numerous procedures are known from the literature such as agroinfection using Agrobacterium tumefaciens or its Ti plasmid, electroporation, microinjection of plant cells and protoplasts, microprojectile transformation and pollen tube transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

8. REVERSAL OF STERILITY

It is apparent, that plants which are made sterile using the above techniques and methods are not desirable per se. Therefore we proposed to use a cascade using molecular elements which will allow the reversal of the engineered sterility to fertility thus permitting maintenance of these plants and their use in F1 hybrid seed production. Design of the reversal mechanism

The reversal mechanism proposed here consists of three separate elements:

a. a chemically switchable promoter

- b. a bacterial operator sequence
- c. a bacterial repressor gene which binds with high affinity the aforementioned operator.

These elements will act in the following way:
5 when restoration of fertility is required (eg in the inbred maintenance plots), plants are sprayed with a chemical. This chemical induces through a chemically- inducible promoter the expression of a bacterial repressor molecule which will bind to
10 operator DNA sequences in the MFS control sequences. This binding will lead to the inhibition of the 'killer' gene function, thus allowing normal pollen formation to take place.

9A. APPLICATION TO F1 HYBRID PRODUCTION

15 Figure 1 outlines the molecular events which will take place when RMS is used in elite plants. Three genetic scenarios are available for the expression of the introduced traits, depending on whether they are destined to act as dominant or
20 recessive genes and on whether expression is arranged to occur in the tissues of the parent sporophyte or gametophyte. These scenarios are summarised in Table I below.

TABLE I
PRODUCTION OF F1 HYBRIDS

Genetic Status of Expression	RMS Genotype			F1 fertile Pollen Load
	Female (MS) Inbred	Male Inbred	F1 Hybrid	
Sporophytic dominant	RMS/+	+/+	RMS/+ or +/+	50% plants (+/+) are 100% fertile
Sporophytic recessive	rms/rms	+/+	rms/+	100% plants are 100% fertile
Gametophytic	RMS or RMS	+ or +	RMS + or +	50% plants are 50% fertile

Where, for example, the RMS components, particularly the "killer gene" functions are expressed as dominant gene(s), it is advisable to utilise RMS in a heterozygous state (RMS/+ in the above-mentioned "Sporophytic dominant" classification). It is implied in this instance that during F1 hybrid seed production, the resulting hybrids are either RMS/+ or +/+ in their genotype. This means that only 50% of the plants (+/+) will be able to contribute to pollen production in the F1 used for grain production. This will be sufficient for most crops,

particularly in maize in which a large amount of pollen is produced.

Importantly, RMS can also be used in the production of other classes of hybrids such as three-way and four-way crosses. In the above-mentioned embodiment using the dominant sporophytic expression, use of the female genotype RMS/RMS in the first cross conveniently generates the genotype RMS/+ on crossing with a +/+ pollinator. The rms/+ genotype then constitutes the male sterile female parent for use in the second cross. (See Table II below).

TABLE II
PRODUCTION OF 3 AND 4 WAY CROSSES

Genetic Status of Expression	RMS Genotype				3 or 4 way Hybrid
	First Female	First Male	Second Female	Second Male**	
Sporophytic Dominant	RMS/RMS	+/+	→ RMS/+	+/+	RMS/+ or +/+
Sporophytic Recessive	rms/rms	ms/rms*	→ rms/rms	+/+	rms/+
Gametophytic	RMS or RMS	RMS or RMS*	→ RMS or RMS	+ or +	RMS or +

* In these embodiments the first pollinator is treated with the chemical switch to restore male fertility.

** The second pollinator may be an inbred (3 way) or or a hybrid (4 way)

9B. APPLICATIONS IN BREEDING PROGRAMMES

In addition to its broad utility in hybrid seed production, the RMS invention is also conveniently utilised in breeding programmes, for example, in enforcing outcrossing in synthetic populations from which new inbred lines are to be derived. Scenarios similar, but not limited to those shown in the above Table for the first cycle in production of three and four way crosses may be employed. Use of the chemical switch facilities subsequent rounds of selfing cycles to produce new and improved genotypes. Molecular screening (RFLP analyses) using DNA sequences derived from the DNA sequences used to facilitate the RMS invention may be conveniently used as probes during inbreeding to ensure that progeny to be advanced have retained all elements of the RMS system. These strategies offer a convenient and efficient procedure to transfer the RMS trait to new germplasm.

The invention will now be described, by way of illustration by the following specific descriptions of various component gene modules which may be utilised. Reference will be made to the accompanying drawings.

25 THE DRAWINGS

Figure 1 is a block diagram of the gene construct of this invention with the plants in the male sterile state;

Figure 2 is a block diagram of the gene construct of this invention with the plants in the male fertile state;

Figure 3 shows the results for total GST activity in roots and shoots obtained 23 and 44 hours after treatment with R25 as described below;

Figure 4 shows the chromatographic separation of the isozymes GST I and GST II.

Figure 5 shows GST I activity present in untreated anther tissue;

5 Figure 6 shows the stimulation of GST II activity after treatment with R25 as described below;

10 Figure 7 shows the results using a stem reservoir technique;

15 Figure 8 shows the results with the application by spray; and,

 Figure 9 is a time course graph generated in the manner described below.

20 Figure 10 shows the structure of vector p35SlacI.

 Figure 11 shows the basic structure of pCG1 and pCG2;

 Figure 12 shows the nucleotide sequence of the maize CAB promoter;

25 Figure 13 is a map of the pAD and pPS1 series of plasmids;

 Figure 14 shows the library screening procedure used for the isolation of maize flower specific clones;

30 Figure 15 shows dot blot analysis of total RNA (4 μ g per dot) extracted from maize tassels of increasing length.

 Figure 16 A, B, C shows in situ hybridisation of maize spikelet sections with pMS14 antisense RNA probes.

 Figure 17 shows the nucleotide and deduced amino acid sequence of MFS cDNA clone pMS10;

 Figure 18 shows the nucleotide and deduced amino acid sequence of MFS cDNA clone pMS14;

Figure 19 shows the nucleotide and deduced amino acid sequence of MFS cDNA clone pMS18;

Figure 20 is a restriction map of the 9kb EcoRI fragment from clone 10/CT8-3;

5 Figure 21 is a restriction map of the 9kb EcoRI fragment from clone 14/17M;

Figure 22 is a restriction map of the 9kb EcoRI fragment from clone 18/CT3;

Figure 23 is a plasmid map of clone pMS10-5;

10 Figure 24 shows the structure of pTAK1, pTAK2 and pTAK3; and,

Figure 25 is a map of clone pMS10-6GUS.

Figure 26 is a map of plasmid pCGS110-UCP;

15 Figure 27 shows the mRNA sequence of mammalian uncoupling protein gene from plasmid pCGS110-UCP (shown in Figure 24);

Figure 28 is a flowchart representation of the generation of a leu2, gal1 yeast strain;

20 Figure 29 is a table showing the effect of addition of galactose on the growth of BET9 and BET27 transformants;

Figure 30 shows results of growth curve analysis of BET9 (Figure 28A) and BET27 (Figure 28B) transformants grown on gly/cas medium over a period of 65 hours in the presence or absence of galactose;

25 Figure 31 is the growth curve analysis of rat UCP in strain BET9 grown on gly/cas medium over a period of 50 hours in the presence or absence of galactose;

30 Figure 32 is the growth curve analysis of rat UCP in strain BET9 grown on raffinose medium over a period of 45 hours in the presence or absence of galactose;

Figure 33 illustrates the construction of plasmid YIP/UCP from pKV49-UCP and YIp5;

Figure 34 is a map of plasmid pGR208 (Figure 32A) and the sequence of oligonucleotides used to mutate the β -subunit gene of F_1 -ATPase (Figure 22B);

Figure 35 shows maps of plasmids pKV49 (Figure 33A) and pKV49-UCP (Figure 33B);

Figure 36 shows schematically the construction of a β -subunit/ β -galactosidase fusion protein;

Figure 37 is a plasmid map of pKV49/BLZ;

Figure 38 is a plasmid map of pMS10-5; and,

Figure 39 is a plasmid map of pBin/MS10-UCP.

THE PLASMIDS

Various gene constructs are described in the following paragraphs and these have been deposited in the National Collection of Industrial & Marine Bacteria in Aberdeen, UK. The dates of Deposition and the Accession Numbers are summarised in Table III below.

TABLE III

Plasmid	Host	Deposit Date	Accession No.
pGAL2	E.coli DH5 α	06 Dec 1988	NCIB 40087
p35SlacI	E.coli TG-2	12 Dec 1988	NCIB 40092
pPS1	E.coli DH5 α	21 Dec 1988	NCIB 40097
pAD18	E.coli DH5 α	21 Dec 1988	NCIB 40096
pMS10	E.coli RR1	09 Jan 1989	NCIB 40098
pMS14	E.coli DH5 α	09 Jan 1989	NCIB 40099
pMS18	E.coli RR1	09 Jan 1989	NCIB 40100

I. THE CHEMICAL SWITCH

This module is exemplified by a chemically inducible gene promoter sequence isolated from a 27kd subunit of the maize glutathione-S-transferase (GST II) gene.

In practice the chemically inducible promoter of the invention will be inserted as a promoter sequence in a recombinant gene construct destined for use in a plant. The construct will then be inserted into the plant by transformation. Expression of protein encoding genes in the construct, being under control of the chemically switchable promoter of the invention, may be controlled by the application of a chemical inducer to the plant.

Examples of effective promoter/inducer combinations are the promoter is the GST II promoter aforesaid and the inducer is N,N-diallyl-2,2-dichloroacetamide (common name: dichloramid) or benzyl-2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate (common name: flurazole)

Chemical inducers which are potential inducers of the GSTII 27kd subunit expression include compounds such as:

1. benzyl-2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate;
2. naphthalene-1,8-dicarboxylic anhydride;
3. 2-dichloromethyl-2-methyl-1,3-dioxolane;
4. 1-(dichloroacetyl)-hexahydro-3,3,8a-trimethyl-pyrrole (1,2-a)-pyrimidin-6(2H)-one;
5. 2,2,5-trimethyl-N-dichloroacetyloxazolidine;
6. 1,3-dioxolan-2-ylmethoxyimono(phenyl)benzene acetonitrile;
7. 4,6-dichloro-2-phenyl-pyrimidine;

8. 2,2-dichloro-[N-allyl-N(1,3-dioxalano-2-methy)] acetamide;
9. 1-(cyanomethoxyimino)benzacetoneitrile;
10. 4'-chloro-2,2,2-trifluoroacetophenone-O-1,3-dioxolan- 2-yl methyloxime;
11. 2,2-dichloro-1-(3,4-dihydro-3-methyl-2H-1,4-benzoxazin-4-yl) ethanone;
12. 3-dichloroacetyl-2,2-dimethyloxazolidine;
13. 4-methoxy-3,3-dimethylbenzophenone;
14. 1-cyclohexyl-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pent-1-en-3-ol;
15. 2,2-dichloro-N-(3-methyl-4-thiazolin-2-ylidene) acetamide;
16. O,O-diethyl-O-phenyl phosphorothioate;
17. 2,2-spirocyclohexyl-N-dichloroacetyl oxazolidine;
18. N-benzyl-N-ethyl-dichloroacetamide;
19. 3-chloroacetyl-4,4-cyclohexane-spiro-2,2-dimethyl-1,3- oxazolidine; and,
20. spirooxazolidine acetamide.

Glutathione-S-transferases (GST) are a family of enzymes which catalyse the conjugation of glutathione via the sulphhydryl group to a large range of hydrophobic, electrophilic compounds. The conjugation results in detoxification of these compounds and in insects and mammals, removal from tissue.

GST enzymes have been identified in a range of crop plants including maize, wheat, sorghum and peas. GST's comprise from 1 to 2% of the total soluble protein in etiolated maize seedlings.

The major isoform of GST can be distinguished in maize tissue. GST I is constitutively expressed and is capable of conjugating glutathione with the

pre-emergent herbicides alachlor and atrazine.
Treatment of maize tissues with chemical safeners
(for example, N,N-diallyl-2,2-dichloroacetamide)
raises the activity of GST I which participates in
the detoxification of the pre-emergent herbicides.

Safener treatment of corn tissue

For treatment of young maize seedlings, seeds
were germinated on moist filter paper. After
germination and growth (up to one week) the safener
N,N-diallyl-2,2-dichloroacetamide (hereinafter
referred to as R25) was added to the water in the
filter paper to give a range of concentrations
(0.003 to 30 ppm) and the seedlings grown for a
further 23 to 44 hours before harvesting of root
and shoot tissue. Figure 3 shows the results for
total GST activity in roots and shoots obtained 23
and 44 hours after treatment as described and
Figure 4 shows the separation of the isozymes GST I
and GST II.

For treatment of maize tassel and anther
tissue, a solution of 800 μ g of R25 was injected
into the node directly below the developing tassel.
Uptake then continued for a further 48 to 72 hours.
Figure 5 shows that only GST I activity was present
in untreated anther tissue and Figure 6 shows the
stimulation of GST II activity after treatment as
described.

Alternatively a 100 ppm solution of R25 was
supplied from a glass reservoir attached to the
exposed stem immediately below the developing
tassel. Figure 7 shows the results using a stem
reservoir technique.

Additionally, R25 was applied as a 100 ppm
spray directly on to the exposed developing tassel.

Figure 8 shows the results with the application by spray.

Both GST proteins have a native molecular weight of approximately 50 kd. As in mammals, maize GST's are dimeric; GST I has apparently identical subunits of 29 kd, whereas GST II is a heterodimer of a 29 kd subunit similar to that found in GST I and a novel 27 kd subunit which is only present in tissue treated with safener except in seedling root where it is constitutively expressed, but still can be induced by safener treatment.

A cDNA and a gene corresponding to the 29 kd subunit of GST I have been cloned previously and sequenced. In addition, a cDNA corresponding to a 26 kd subunit of a third, minor component of GST activity in maize seedlings (GST III) has been previously cloned and sequenced.

Enzyme Assay

Enzyme activity was measured spectrophotometrically at 340nm using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The reaction buffer contained 0.1M EDTA, 0.001M CDNB and 0.0025M glutathione.

Preparation of extracts and enzyme purification

Tissue was homogenised in 0.05M Tris.HCl, pH 7.8; 0.001M EDTA; 0.001M DTT; and 7.5% polyvinylpyrrolidone in a pestle and mortar, at 4°C, and centrifuged at 30,000g to obtain a crude extract.

Separation of the GST isoforms from the crude extract was achieved as follows: the crude extract was applied to a DEAE Sepharose column and washed

with 0.01M Tris.HCl, pH 7.8; 0.001M EDTA; and
0.001M DTT. The bound GST was eluted with 0.3M
potassium chloride. Fractions containing GST
activity were combined and desalted using PD10 gel
5 filtration columns. Separation of the GST I and
GST II isoforms was achieved by FPLC on a mono-Q
column and a zero to 0.4M potassium chloride
concentration gradient.

Pure samples of GST I and GST II were obtained
10 by applying desalted fractions of GST I and GST II
from the FPLC to a glutathione-S-sepharose affinity
column equilibrated with 0.05M phosphate buffer at
pH 7.3. After washing with buffer, bound GST was
eluted with 0.005M glutathione.

15 SDS-PAGE (17.5%, 30:0.174 acrylamide:
bisacrylamide) of GST I or GST II was achieved by
concentrating pure GST samples using Amicon
Centricon 10 Microconcentrations (Trade Mark),
denaturing samples in mercaptoethanol containing
20 Laemmli buffer, and staining the gels with
Coomassie Blue.

Generation of antibodies to the enzyme

Sufficient protein to enable the immunisation
of rabbits is obtained by pooling the isolated
25 enzyme subunit isolated as described above from a
number of separate experiments. The 27 kD GST II
polypeptide is subsequently purified to apparent
homogeneity by electroelution from polyacrylamide
gel slices. Antisera are prepared against the 27 kD
30 polypeptide. The immunisation of rabbits is carried
out essentially according to Mayer and Walker
(1978).

N-terminal sequence analysis

The amino terminal sequence of the intact 27
kd subunit of GST II or partial proteolytic
cleavage products was determined by sequential
5 Edman degradation and subsequent amino acid
analysis by HPLC.

Time Course

A time course experiment was carried out to
examine the expression of GST's after safener
10 treatment. A 30 ppm solution of R25 was applied to
three-day old seedling roots and tissue harvested
after various time intervals following safener
treatment. Samples were tested for GST activity
using the enzyme assay described above. The
15 results of this experiment are presented
graphically in Figure 9.

Synthesis of cDNA libraries

The time course experiments revealed a peak of
GST expression at 48 hours after treatment with
20 safener. Therefore, two cDNA libraries were
constructed from RNA extracted from tissue at 24
and 48 hours after safener treatment. To ensure
that the induction procedure had been successful, a
one gram sample of 24 hour induced tissue was taken
25 and assayed for GST II. This experiment revealed
that the tissue used to construct the cDNA library
had indeed been successfully induced as GST II
accounted for 45.5% of the total GST activity.

Double-stranded cDNA was prepared from oligo
30 dT-cellulose-purified RNA by a method employing
RNaseH and E.coli DNA polymerase I in the synthesis
of the second strand, without prior purification of
single-stranded cDNA (Gubler and Hoffman, 1983).

Screening cDNA libraries with antisera to GST I and GST II

5 In order to identify a cDNA clone encoding maize tassel GST enzyme, bacteriophage from the amplified cDNA library are screened with anti-maize GST enzyme serum. The clones producing the strongest signals are re-screened.

Screening cDNA libraries using oligo probes

10 Mixtures of synthetic oligonucleotides based on the amino acid sequence determined above were prepared by phosphoramidite chemical synthesis. The 5' ends of the oligonucleotides were labelled using polynucleotide kinase as described in the literature.

15 Approximately 40,000 phages containing cDNA were amplified on plates and transferred to nitrocellulose. The filters were hybridised to oligonucleotide probes at temperatures of from 2 to 5°C below the melting temperature calculated for the lowest melting point probe in the mixture. Hybridising plaques were selected and rescreened through two or more rounds exactly as described above but at lower densities

Isolation of cDNA gene sequences by the PCR method

25 cDNA or DNA sequences are isolated from the libraries described using oligo primers based on the amino acid sequence obtained from partial proteolytic cleavage or in the case of genomic DNA, primers based on cDNA sequence determined previously.

Characterisation and sequence analysis of GST cDNA clones

The isolated cDNA is characterised and subjected to sequencing by one or more of the

standard available techniques.

Isolation of genomic sequences

5 An existing genomic library of fragments of total maize DNA cloned into λ EMBL3 is used to isolate clones that hybridise to the cDNA clones isolated as described above.

Alternatively, the PCR method described above may be used to selectively amplify and clone gen fragments. GSTII genes and ther promoter sequences
10 can then be isolated, and characterised using established techniques. It can be demonstrated that the GSTII promoter sequences mediate safener-induced gene activity by fusing them to marker genes like GUS and CAT, and testing then in
15 transgenic plants.

II. THE REPRESSOR AND OPERATOR SEQUENCES

This module regulates plant gene expression. More particularly, the module regulates plant gene expression by the use of repressor molecules of
20 bacterial or lower eukaryotic origin.

Traditionally, the improvement of crop plant species involves the introduction of desired traits by genetic crosses. However, although these breeding techniques are highly successful, they
25 provide no means of controlling the expression of the newly acquired traits. Recent advances in technology are now allowing the genes responsible for determining plant structure and the productivity and quality of the crop to be
30 identified and isolated. A major aim in the field of improvement is therefore to be able to manipulate complex developmental processes genetically in order to improve crop performance. Essential to this objective is the determination of

strategies which allow the expression of specific plant genes to be regulated at will.

The ability to control the expression of traits according to circumstances has many important applications, such as control of insect resistance genes, determination of plant height and timing of flowering and the control of plant fertility. Additionally, the ability to switch genes on or off at will, without disturbing plant physiology or environment, would be an invaluable tool in the study of plant genetics per se.

Currently, the production of seed for hybrid crops such as maize involves the laborious and expensive process of hand or mechanical emasculation of the parent plants in order to prevent self-pollination. Such emasculation can, however, be controlled genetically by making use of a trait known as cytoplasmic male sterility (CMS) which has been observed in a wide variety of crop species. CMS interferes with male gametogenesis, resulting in the inhibition of pollen formation, but does not normally affect female fertility. Consequently, "male-sterile" plants are able to set seed, such seed resulting only from cross-pollination. The ability to control the expression of these genes would allow male gametogenesis to be inhibited in the production of hybrid crop seeds without the need for expensive emasculation processes, while still allowing genetic improvement of the male parent by conventional breeding programmes.

Control of gene expression in both prokaryotes and eukaryotes relies primarily on the interaction of regulatory proteins with specific DNA sequences.

Depending on the nature of these interactions, transcription from the cognate promoters may either be repressed or activated. Indeed, in some cases the same protein may either reduce or enhance transcription according to the nature of the contacts made. Furthermore, the ability of some regulatory proteins to bind their target sequences is modulated by the binding of ligands or by specific proteolytic cleavage. Such mechanisms may be exploited in order to include inducibility amongst strategies for plant gene regulation.

The best characterised regulatory systems are those of bacteria in which the interactions between the DNA-binding proteins (repressors) and the target DNA sequences (operators) are understood in great detail. A comparison of the best understood systems, including repressor and cro proteins of bacteriophage λ and 434, the LacI repressor and the catabolite gene-activating protein (CAP), reveals several factors in common. These regulatory proteins bind as dimers or tetramers to short operators that exhibit a high degree of dyad symmetry. In most cases the domain responsible for DNA-recognition, which is separate from that concerned with oligomerisation of the monomers, contains a conserved helix-turn-helix structure. A specific helix within this structure in each monomer, the recognition helix, is aligned with the major groove of the DNA and only if specific contacts are formed between the amino acids of this recognition helix and the bases of the adjacent DNA can a functional repressor/operator complex be

formed. Such interactions are highly specific, and the high-affinity complexes are formed with extremely rapid kinetics.

5 The knowledge of mechanisms by which gene expression is regulated in eukaryotes is much less detailed. In yeast and mammalian cells a large number of binding sites for putative regulatory proteins have been identified in promoter sequences, and in some cases the proteins
10 responsible have also been isolated. However, only in a few instances are the molecular details known of the protein-DNA interactions and the mechanism by which transcription is regulated.

15 In plants, regulation of gene expression is understood at only a rudimentary level. Several regulatory elements have been identified in promoter sequences, and some regulatory proteins examined at a preliminary level. However, such proteins have yet to be isolated and the details of
20 the mechanisms involved elucidated.

Eukaryotic regulatory systems appear to exhibit a greater diversity of structure and a higher degree of complexity than their prokaryotic counterparts. For instance, control of
25 transcription from eukaryotic promoters is thought to involve the interaction of many proteins (perhaps in the order of tens) with the regulatory DNA. Furthermore, at least three different protein structures (the helix-turn-helix, the zinc-finger
30 and the leucine zipper) have been implicated in the specificity of DNA-binding by various eukaryotic regulatory factors.

DNA-binding proteins constitute a class of proteins characterised by their ability to bind to DNA of genes to give the effect of either repressing or activating the gene to which they bind. Unless the context otherwise requires, such DNA-binding proteins will hereinafter be referred to for convenience simply as "repressors".

This module, then, is a recombinant plant gene comprising a repressor gene of bacterial origin and a promoter which operates in plants for driving expression of the repressor gene, said gene encoding a repressor protein capable of interaction with an operator sequence associated with a selected target plant gene so that on expression of the repressor protein expression of the target plant gene is inhibited.

A vector, designated p35SlacI, containing the said DNA, which has been deposited in an E.coli, strain TG-2, host with the National Collection of Industrial and Marine Bacteria Limited, Aberdeen, United Kingdom, on 12th December 1988, under the Accession Number NCIB 40092.

A suitable plant transformation vector comprises Agrobacterium tumefaciens, harbouring the plasmid aforesaid.

In a specific embodiment of this module a bacterial lacI operator system is utilised to regulate gene expression. Lac repression can be relieved by iso-propyl thiogalactoside (IPTG) and other sugar analogues.

Specific Examples relating to this module will now be given.

EXAMPLE(1) Construction of plants expressing
the lac repressor

5 Vectors were constructed which express the
lacI gene from either the constitutive CaMV 35S
promoter found in vector pJR1 or from the green
tissue- specific promoter, the maize CAB promoter.
However, the bacterial repressor can be expressed
from any plant promoter expressed in other parts of
10 the plant, thus allowing control of plant gene
expression in any specific part of the plant.

(1.1) Modification and insertion
of the lacI repressor gene into pJR1

The lac repressor (lacI^Q) is available on
15 plasmid pMJR 156. In order to express this gene in
plants, the translation initiation codon (GTG) had
to be changed into ATG. In addition it was
opportune to create a suitable restriction enzyme
cleavage site for cloning of this gene into a plant
expression vector. At the 3' end of the lacI there
20 are suitable restriction sites (HindIII and PstI)
for insertion into plant expression vectors. In
order to create suitable restriction sites at the
5' end, the following experiments had to be
25 performed:

(a) A Cfr 10 restriction site is located at
position 134. pMJR was cut with Cfr 10 and a
synthetic DNA fragment which reconstitutes the
N-terminus of the lacI gene, the altered
30 translational start codon ATG, a plant consensus
sequence for efficient translational initiation and
a BamHI restriction site were inserted into pJR1.
The sequence of this synthetic fragment was:

BamHI consensus

GATCC AACAAATGGCT AAACCAGTAACGTTATACGATGTCGCAGAGTAT G

G TTGTTACCGA TTTGGTCATTGCAATATGCTACAGCGTCTCATA CGGCC

Cfr 10

5 pJR1 was cut with BamHI and PstI. The synthetic fragment described above, and the Cfr10 to PstI fragment containing the lacI gene were ligated together with the cut vector pJR1 under standard conditions. The ligation mix was transformed into

10 E.coli TG-2. Recombinants were selected on kanamycin-containing plates. They were characterised by DNA sequence analysis. The construct was designated p35SlacI.

(b) The PCR (Polymerase Chain Reaction) as described by Saiki et.al., Science, 239, 487-491) was utilised to introduce the changes at the 5' end of the lacI gene while keeping the sequence at the 3' end. Two oligonucleotides were hybridised to pMJR 156. The sequence of the oligonucleotides

20 were:

(i) from the 5' end of the gene

BamHI consensus

GAGAGTCAATTCAGGGT GGATCC AACAAATGGCT
AAACCAGTAACGTTATACG

25 (ii) from the 3' end of the gene
CGTTGTAAAACGACGGCCAGTGCC

The PCR reaction was carried out under the prescribed conditions. The product was cut with BamHI (at the newly introduced site) and PstI. The

30 resulting fragment was cloned into pJR1 cut with BamHI and PstI. Recombinants were identified by hybridisation and restriction analysis using

standard protocols. One of the resulting clones was characterised by DNA sequence analysis.

Both of the methods (a) and (b) gave the same construct, designated p35SlacI. Figure 10 shows the structure of vector p35SlacI.

(1.2) Replacement of the CaMV 35S promoter
with maize CAB promoter

In order to demonstrate the general utility of the Lac repressor/operator system in plants, we have constructed an expression vector which will allow inducible and tissue-specific lacI expression in plants. For this work, we have used the promoter of the gene encoding the light-inducible maize chlorophyll a/b binding protein (CAB).

The construction of this vector was achieved by replacing the CaMV promoter in p35SlacI with the maize CAB promoter, the DNA sequence of which is given in Figure 12 herewith, which is found in vector pCAB48.1. The CaMV promoter was removed by restriction of p35SlacI with EcoRI and BamHI using standard conditions. The CAB promoter was isolated from pCAB48.1 by restriction with XbaI and Sau3A using partial restriction conditions for Sau3A. This promoter fragment was then inserted into the promoter-less p35SlacI. This vector, designated pCABlacI, has been characterised by restriction mapping and DNA sequence analysis.

(1.3) Transformation of tobacco plants

The expression modules from the vectors described above were transferred to BIN19 and then to tobacco using leaf disc transformation following standard protocols. The plasmids were transferred to Agrobacterium using triparental mating. Agrobacteria were purified, and used in leaf disc

transformation experiments. Thirty-seven plants containing the CaMV-lacI expression module and thirty-eight plants containing the BAB-lacI construct were regenerated and analysed for the relative expression of lacI.

1.4 Analysis of transgenic plants for lacI expression

The expression of the lacI gene was monitored using Western analysis of extracted proteins. Extracts were prepared, proteins recovered on polyacrylamide gels and prepared for Western analysis. The analyses confirmed the expression of the lacI gene construct in the transformed plants. Different levels of lacI gene expression were observed in different independent transformants. The results for plants transformed with the CaMV-lacI construct are given in the following Table IV.

TABLE IV	
Plant Sample	Lac Expression (Band Intensity)
L1	-
L2	++
L3	-
L5	-
L7	-
L8	+++
L9	+
L10	+
L11	-
L13	+
L14	++
L15	-
L16	+++
L18	-
L22	+/-
L23	-
L24	++
L25	+
L26	+++
L27	+++
L28	-
L29	++++++
L31	++
L32	++++
L33	+/-
L34	+/-
L35	++
L37	+
<p>- → ++++++ indicates band intensity of Lac repressor from Western blots.</p>	

terms of the Budapest Treaty, in an Escherichia coli, strain DH5 α , host, with The National Collections of Industrial and Marine Bacteria Limited, Aberdeen, United Kingdom, on 21 December 1988, under the accession Number 40096.

The plasmid pPS1 has been deposited, under the terms of the Budapest Treaty, in an Escherichia coli, strain DH5 α , host, with the National Collections of Industrial and Marine Bacteria Limited, Aberdeen, United Kingdom, on 21 December 1988, under the accession Number 40097.

This procedure is also applicable to protein molecules which lead to an increase in gene activity, particularly the selection of repressors/activator proteins which respond to specific chemicals. Binding domains for these chemicals can be selected and specifically manipulated to allow the generation of specific protein/DNA effect chemical combinations which are of use in biotechnology, for example as a chemical switch package enabling the controlled regulation of plant genes by application of an exogenous chemical inducer.

Mutations which affect both repressors and operators occur in vivo. It has been shown that repressors which have altered DNA recognition specificities can be engineered in vitro. The procedure, then, depends on the ability of rare repressor mutants to switch off a conditionally lethal gene by binding at pseudo-operator sequences which the native repressor cannot recognise.

One embodiment of the invention will now be described, by way of illustration, in the following example, with reference to the accompanying drawing

2. Insertion of the lac operator into target genes

(a) The maize CAB promoter

The maize CAB promoter can be found in plasmid pCAB48.1 and we have found that this promoter can drive expression of foreign genes in a transient tobacco expression system and in stably transformed plants. This gene, therefore, is an excellent target to demonstrate control through lacI as high levels of expression can be obtained both in vitro and in vivo. Secondly, the CAB promoter from other systems (wheat, pea and tobacco) have been extensively analysed in detail and reported in the literature. The published information facilitates the selection of suitable sites for operator insertion. Thirdly, pCAB48.1 is a maize promoter and the use of this system is important to demonstrate the applicability of this invention to monocotyledonous plants such as maize, wheat, barley and sorghum.

(b) Insertion of the lac operator into the maize CAB promoter

Relatively little work has been reported concerning the characterisation of the important cis-acting elements of the CAB promoter. Therefore, a computer search comparing consensus upstream regulatory elements (UREs) of several plant genes against the CAB promoter was carried out. As anticipated, numerous putative UREs were found in both strands of the CAB promoter. A number of potential sites for operator insertion were selected.

1. 5' of the CAAT box;
2. Between the CAAT and the TATA box;
3. Around the TATA box;

4. Between the TATA box and the transcription start point; and,
5. Between the transcription start point and the translation start point.

5 Two methods were used to insert the lac operator into the maize CAB promoter:
(1) insertion into naturally occurring restriction sites;
(2) using PCR to introduce operators at selected
10 sites.

 These methods were used to insert lacI operators into the selected sites.

Method (1)

15 Analysis of the promoter sequence shows that this region does not contain many unique restriction sites. However, two sites can be made available by simply recloning the promoter region into various vectors.

(a) Insertion between TATA and TSP

20 The restriction enzyme PvuII recognises a single site within the 2.8 kb PstI fragment containing the CAB gene. The site lies between the TATA element and the transcription start point (TSP) of the CAB promoter. However, the vector
25 pCAB48.1 contains numerous PvuII sites (within pUC19). Therefore, the 2.8 kb PstI fragment was cloned into the standard cloning vector pAT153 (which lacks a PvuII site) to give pCABP1.

30 Operator sequences were inserted into the unique PvuII site within pCABP1. After sequencing, it was possible to determine which clones contain single and tandem operator insertions. The synthetic symmetric lac operator required for this work is shown below and is an 18

base pair palindrome that is analogous to a mutant operator which binds lac repressor eight times more strongly than the wild-type operator.

lac operator-1

5 5' -ATTGTGAGCGCTCACATT- 3'

(b) Insertion upstream of the CAAT sequence

The method used was as follows:

(i) pCAB48.1 was digested with HindIII, which cuts outside the promoter region and within pUC18, and BglII, which cuts downstream of the unique NcoI site and within the coding region. This gave a fragment with a unique SphI site upstream of the CAAT moiety;

(ii) pUC18 was digested with HindIII and BamHI and the promoter fragment from (i) above was inserted to give pCABP2. Digestion of pUC18 with BamHI removes the single SphI site from the polylinker. Therefore, pCABP2 contains a unique SphI site into which operators can be inserted.

20 The operator used in this procedure had the sequence:

lac Operator-2

5'-ATTGTGAGCGCTCACAATCAT G-3'

3'-GTACTAACTCTCGCGAGTGTTA-5'

25 It is important to note that in approaches (a) and (b) the operator sequences are not inserted directly into any putative regulatory elements although promoter activity is likely to be affected when the sequences are inserted elsewhere.

30 Method 2

As shown above, operator sequences can be inserted into two available restriction sites. Insertion into other sites requires other methodologies.

Insertion between TSP and ATG codon

This can be effected using PCR. Since a unique PvuI site lies close to the TSP region, it is used as a reference point for subcloning purposes. The starting material for the PCR reaction is pCABP1, that is, the pAT 153 CAB promoter vector constructed as described above. An oligonucleotide overlapping the PvuII site and containing no alterations was used to prime the reaction from one end:

CAB Oligonucleotide-1PvuII

5'-GG CAGCTG CTGTGTTCTGTTATGAC-3'

The second oligonucleotide overlaps the NcoI site and contains the operator sequence shown below.

CAB Oligonucleotide-2NcoIOperator 1

5'-GATAG CCATGG TGGCGGCAGCCATGTCG ATTGTGAGGCGCTCACAAT
--ATCAGATCGTAGCTCCTTCTGATGC-3'

CAB Oligonucleotide-3NcoIOperator 1

5'-GATAG CCATGG TGGCGGCAGCCATGTCG ATTGTGAGGCGCTCACAAT

Operator 2

-ATTGTGAGGCGCTCACAAT ATCAGATCGTAGCTCCTTCTGATGC-3'

Following the PCR reactions, the newly synthesised DNA is cleaved with PvuII and NcoI. The fragment is then transferred to similarly digested pCABP1 and sequenced.

A slightly different approach which eliminates the intermediate cloning step into pCABP1 may also be used. This involves using an oligonucleotide which overlaps the unique XbaI site in the CAB promoter together with the operator nucleotides outlined

previously. Digestion of PCR DNA with XbaI/NcoI results in a fragment which can be directly cloned into pCG1 and pCG2. However the XbaI to NcoI fragment from the PCR reaction is much larger than the PvuII to NcoI fragment obtained from the previous strategy.

Operator insertion between the CAAT and TATA

This is effected using PCR.

CAB Nucleotide-4

10

XbaI

5'-CCCAAACAG TCTAGA TATGTTTCTC-3'

CAB Nucleotide-5

PvuII

Operator

15

5'-CAGAACACAG CAGCTG CCTTTTATAC ATTGTGAGCGCTCACAAT-
-AGTTGGGTTTGGATAGCAGGTCATC-3'

CAB Nucleotide-6

PvuII

Operator 1

5'-CAGAACACAG CAGCTG CCTTTTATAC ATTGTGAGCGCTCACAAT-

Operator 2

20

-ATTGTGAGCGCTCACAAT AGTTGGGTTTGGATAGCAGGTCATC-3'

Following PCR, DNA is digested with XbaI and PvuI and cloned into similarly digested pCABP1. Clones are again characterised by sequencing and any appropriate DNAs are digested with XbaI and NcoI and cloned into pCG1 and pCG2. The basic structure of these vectors is shown in Figure 11.

25

The CAMV 35S promoter

We have found that a promoter-less 35S vector is an excellent receptor for the insertion of activating sequences. The lac operator can be inserted into this vector, p-Δ-35S, and once inserted the 35S enhancer is cloned 5' upstream of the lac operator.

30

(3) Control of gene expression by lac repressor

(a) Control of target gene expression in a
transient expression system

Plants which express lacI constitutively
5 transformed with p35lacI may be prepared from
protoplasts and, using methods described above) they
may be tested for expression of the lacI protein.
The target gene constructs may then be introduced into
the protoplasts using standard methods and protocols.
10 Protoplasts from untransformed plants can serve as
control. Further control may be provided by
protoplasts from plants expressing the GUS marker gene
under the control of the CAB promoter without the
operator insertions.

15 (b) Induction of gene expression using IPTG

IPTG can be used to overcome repression by the
lac repressor. Thus, there is formed a switchable
gene system.

(c) Modulation of expression of the target gene

20 Lac repressor/operator interactions can
down-regulate marker gene expression in plants to
different levels. This is an important effect in
that there may be situations where a different degree
of down-regulation may be required.

25 (d) Control of target gene expression in stably
transformed plants

Having shown, as described above, that the
lac-repressor can down-regulate CAB promoter driven
GUS expression in protoplasts, suitable operator
30 insertion constructs may be transferred to tobacco
plants by the methods described above. The
regenerated plants may be crossed with the lacI
expressing plants described above, which express the

lac repressor under control of the constitutive CaMV35S promoter.

Plants may also be constructed which express the lacI gene under control of the light-inducible maize CAB promoter. The expression of the lacI gene in these plants will then be light-inducible. These plants may be crossed with plants which contain the GUS marker gene from the CaMV promoter containing the lacI operator insertion.

10 Insertion of multiple operators into the CAB promoter

Using similar techniques as described for insertion of single operators, multiple operators can be inserted into the target promoter. This can either be by the insertion of multiple copies of the operator at one site, or the combination of fragments of the promoter in which the operator is inserted at different positions in the promoter, this yielding vectors in which the multiple operators are located at multiple locations in the promoter.

20 SECOND EMBODIMENT

In this embodiment the method of regulating gene expression comprises locating within or inserting into a gene a pseudo-operator sequence, and providing a mutant regulatory gene encoding a repressor having an amino acid sequence which binds to the pseudo-operator.

Cells containing interacting repressor and operator genes may be isolated by a method comprising preparing a recombinant plasmid containing (1) the Escherichia coli lac operon, which includes the lacZ, lacY and lacA genes, and (2) a gene encoding a repressor protein, inserting said plasmid into a bacterial host and culturing same in the presence of

ortho- or para-nitrophenyl-1-thio- β -galactosidase, whereby the growth of cells in which expression of the lac gene is not repressed by the said repressor molecule is inhibited whereas the growth of cells in which repressor/ operator binding occurs is not so inhibited, and recovering cells displaying non-inhibited growth characteristics.

Mutant repressors may be used or an exogenous potential pseudo-operator may be inserted within the operator region of the lac operon. The exogenous potential pseudo-operator is preferably of plant origin.

A convenient bacterial host is Escherichia coli.

Thus, we have a means for altering the repressor of gene expression enabling genes to be inactivated. Pseudo-operators are DNA sequences which maintain the overall dyad symmetry of an operator but which contain different constituent bases. Computer analysis of known DNA sequences of the French bean GPAL2 gene among many others, and promoter and the mammalian c-myc genes, has revealed a number of possible pseudo-operators to different bacterial repressors.

[The plasmid pGAL2 has been deposited in Escherichia coli strain DH5 on 6th December 1988 with the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number NCIB 40087.]

Thus, it is probable 'pseudo-operator' sequences can be found in all genes. In general, then, a pseudo- operator is a DNA sequence present at a suitable position in a gene, including a plant gene, at which repressor binding will lead to inhibition of gene expression.

The plasmid pAD18 has been deposited, under the

which shows a map representing two series of plasmids designated pPS and pAD and variants.

EXAMPLE

5 We demonstrate the selection system of the invention on repressor phage 434. However, in principle any other repressor can be adapted for this selection system.

1. The selection system

10 We have designed a selection system that can be used for the selection of mutants in a wide range of repressor-operator systems. The selection system comprises a set of plasmids and the appropriate *E. coli* hosts, as well as a suicide substrate selection protocol adapted for the plasmids and hosts.

15 In its final form the system depends on the ability of rare repressor mutants to switch off a conditionally lethal gene through the binding at a 'pseudo-operator' which the wild type repressor cannot bind. The selection system described below contains
20 features which maximise the frequency of such repressor mutants to be identified in the final population of cells.

The selection procedure is based on the lac operon of *Escherichia coli* and the use of the suicide
25 substrate para-nitrophenyl-1-thio- β -D- galactoside (TPNPG). The lac operon (which contains the three genes lacZ, lacY and lacA) is controlled by the binding of LacI repressor to an operator sequence, lacO, situated between the transcription start site
30 and the lacZ gene. The lacY gene product, lactose permease, is responsible for the active uptake of lactose and related compounds into the cytosol where they are hydrolysed by β -galactosidase (the lacZ gene product) to form galactose and glucose.

The positive selection system exploits the discovery that the growth of cells expressing the lacY gene is selectively inhibited in the presence of TONPG or TPNPG, presumably through the waste of metabolic energy on its transport. The selectivity of these compounds has been shown to be enhanced when succinate is used as the carbon source.

The rationale behind the selection is based on the ability of 434 repressor to bind the pseudo-operator sequences inserted in the promoter driving expression of the lac gene cassette. In the absence of a 434 repressor/operator complex, the lac operon will be expressed and, in the presence of TPNPG, will result in cell death. Conversely, in the presence of a complex, the LacY permease will not be expressed, and the suicide substrate TPNPG will be unable to enter the cell. Consequently, in the final analysis, a pseudo-operator chosen from the natural sequence of the target plant gene will be cloned into the SalI site and combined with a pool of genes encoding 434 repressors in which certain amino acids in the $\alpha 3$ helix are randomly substituted. Only those cells expressing mutant repressor that is able to bind the pseudo-operator, and consequently repress lacY expression, will be selected in the presence of TPNPG.

2. The plasmids

2.1 Construction of pAD18 and Derivatives

A series of plasmids have been developed for use in these experiments. The prototype of these is pAD18, a map of which is shown in Figure 13. This vector is based on a replicon from pSC101, which is known to be stably maintained in E. coli, and to have a low copy number. This is important as overexpression of DNA binding proteins may have

deleterious effects on the growth of the host. If this is a problem in some experiments, it is advisable to transfer genes contained on the pAD18 to a bacteriophage vector for insertion into the bacterial genome as a single copy gene.

pAD18 has a kanamycin selectable marker for maintainance in *E. coli* strains.

pAD18 also contains the lac operon. The lacZ and lacY genes are present under the control of the lac promoter/operator. Into the lac operator, a Sall restriction site has been engineered which is used for insertion of the 434 operator, or in derivatives of pAD18, mutant 434 operators or selected 'pseudo-operators'. This site has been positioned in such a way that it will not interfere with expression of the lac operon from the lac promoter through steric hinderances when sufficient repressor is synthesised to bind to the operator cloned into that site. Those bases were changed into Sall restriction sites which are known not to be involved in contact with RNA polymerase. Thus the lac operon expression will be manipulated under the control of the 434 repressor. pAD18 which contains the wild-type 434 operator is thus the prototype of this series of plasmids.

pAD18 contains a tetracycline resistance gene, into which a wild type 434 repressor gene under the control of the lacUV5 promoter can be inserted for high level of expression. This vector is called pPS1. Further derivatives are described below.

In another vector, the 434 repressor has been modified such that a KpnI and NotI site has been introduced at either side of the DNA binding helix whilst the native amino acid sequence in this region has been preserved. It is thus be possible to insert

into this 434 repressor gene random oligonucleotides which when expressed will generate 434 repressor molecules which express altered DNA binding domains. The selection system using a suicide substrate will then permit the selection of those 434 mutant repressors which bind to the 'pseudo-operator'. In some circumstances this may also provide the selection pressure for isolation of repressor mutants. However, the system as it stands is dependent on expression/repression of the lacY permease for the isolation of repressor mutants.

Thus, there are convenient cloning sites in pAD18 and its derivatives for insertion of operators or repressor genes. Operators can be cloned into precursor vectors of pAD18, especially pRW283, from which the operator containing EcoRI to PstI fragment can subsequently be excised and cloned into EcoRI and PstI digested pAD18 (see Figure 13).

One objective was to show that the expression of the lac operon carried by the plasmids described above could be controlled by 434 repressor/operator interactions. To demonstrate this, three plasmids were constructed in which the 434 operators carried by pAD16, 17 and 18 were combined with the wild-type 434cI gene on pAD15.2. The large XhoI/SstI fragment (9.4kb) from pAD15.2 was purified and ligated to the small XhoI/SstI fragment (3.7kb) purified from pAD16, 17 and 18 to form plasmids pPS2, pPS3 and pPS1, respectively. Restriction analysis of plasmid DNA isolated from several transformants from each ligation showed that all pPS plasmids had the expected overall structure. The structure of these plasmids is shown in Figure 13.

The integrity of the operators carried by the pAD

and pPS plasmids was checked by sequencing. Initially this was achieved by isolating the approximately 200 base-pair EcoRI/PstI fragment, which carries the whole of the lac promoter and the 5' end of the lacZ gene, from each of the pAD and pPS plasmids and subcloning them into the polylinker of M13mp18. Single-stranded template was purified and sequenced according to standard protocols. Alternatively, this laborious subcloning procedure was circumvented by using plasmid sequencing. These analyses showed that the appropriate 14mer operator sequence was present at the SalI site in all relevant plasmids. The presence of other salient features of the lac promoter was also confirmed.

2.2 pPS plasmids encode functional 434 repressor.

To visualise the 434 repressor produced by pAD15.2 and the pPS plasmids, total protein extracts were prepared from mid-log cultures grown under selective conditions. Following polyacrylamide gel electrophoresis and Coomassie brilliant blue (G250) staining, no proteins corresponding to the size of the repressor could be observed specifically in strains containing the 434cI gene. However, other experiments have shown that 1 μ g of purified repressor is only just visible using this relatively insensitive technique. Therefore, to detect 434 repressor in the amount of extract used an expression level of at least 1% total cell protein would be required. The background of other similar-sized proteins also makes detection difficult. Consequently, the much more sensitive Western blotting technique was used. The primary antibody required to detect 434 repressor by Western blotting was prepared by injecting rabbits with

purified intact 434 repressor. The specificity of this polyclonal antiserum was demonstrated using purified repressor and extracts of E.coli strains harbouring the 434cI gene. At low dilutions of antiserum several proteins from bacterial extracts, including 434 repressor, were detected. However, further dilution of the antiserum resulted in only 434 repressor remaining detectable, the maximum specificity being observed at dilutions of 1/10000 to 1/20000.

The sensitivity of Western blotting using this antibody preparation and the horse-radish peroxidase conjugate detection technique was assessed by "spiking" crude cell extracts of 6300Δlac4169, which contains no 434cI gene, with various amounts of the purified repressor. Under standard conditions, 5ng of repressor in 50μg of extract could readily be detected, this sensitivity corresponding to an expression level of 0.01% total cell protein.

Use of the same primary antibody in Western blots of 6300Δlac4169 strains carrying the test plasmids showed conclusively that cells harbouring pAD15.2, pPS1, pPS2 and pPS3 all synthesised 434 repressor. Determination of the relative intensities of the bands obtained, using a scanning densitometer, showed that all four strains contain approximately 0.4% total cell protein as 434 repressor.

Finally, the ability of the 434 repressor to bind wild-type operator sequences was determined in a functional assay using bacteriophage 434cI, 434vir and λcI. In the life cycle of these phage, the binding of the appropriate repressor to operators within the promoter P_R represses transcription of the genes

responsible for cell lysis. Consequently, cells which endogenously synthesise cI repressor are immune to lysis by the corresponding phage due to the inhibition of lytic gene expression by the already existing repressor. Since the cI mutant phage are unable to synthesise repressor, the lytic phenotype after infection by these phage is diagnostic for the absence of repressor in the host cell. The P_R operators of vir mutant phage have a reduced affinity for repressor, with the consequence that at low levels of endogenous repressor this phage is lytic, whereas at higher repressor concentrations super-infection is inhibited.

The results of cross-streaking these phage with the test strains show that cells harbouring the pPS plasmids are immune to super-infection by both 434cI and 434vir, but are sensitive to λ cI. Strains carrying other pAD plasmids were sensitive to all three phage. This clearly indicates that cells carrying the pPS plasmids synthesise high levels of 434 repressor that is functionally able to bind operator and inhibit transcription from P_R . Furthermore, the specificity of this repressor/operator interaction is demonstrated by the inability of 434 repressor to bind the operators of λ cI, which have a different sequence to those of 434, resulting in cell lysis.

In summary, all three pPS plasmids were determined to be of the correct construction, to carry the expected 434 operator sequence and to synthesise functional 434 repressor.

2.3 VECTOR IMPROVEMENT

As indicated below, a proportion of the population of pAD and pPS plasmid-containing strains

form white colonies upon selection. Furthermore, it has been observed that if strains containing these plasmids are kept on selective media for several months, sub-culturing when necessary, the proportion of white colonies in the population increases.

It is presumed that these white colonies carry plasmids in which part of all of the lac operon is mutated or deleted. The usual method of minimising such problems is to use a recombination-deficient strain. However, the combination of the Δ lac4169 and recA56 alleles renders the strain inviable in the presence of TPNPG, the reason for this being unclear. Therefore, attention has turned to the probable source of the recombinatory events. It is noted that the promoters expressing the lac operon and the 434 repressor gene in the pPS plasmids are both derived from the lac promoter and consequently the sequences are very similar. Recombination between these sequences would result in the deletion of the lac operon, given that the origin sequences and the kanamycin-resistance gene must remain under the selective conditions imposed.

During the construction of pAD15.2, the 434cI gene was transferred from plasmid pRP42 on a 1kb Sau3A fragment. The stop codon of the 434 repressor reading frame coincides with the Sau3A site at the right hand side of this fragment, consequently the gene cloned into pAD15.2 carries no transcriptional termination signals. Furthermore, this Sau3A fragment also carries a remnant of pBR322, including the ampicillin-resistance gene promoter. Therefore, to rectify these problems sequences both upstream and downstream of the 434cI coding sequence were altered. The region upstream was replaced with the tryptophan promoter and

a consensus Shine-Dalgarno sequence using appropriate oligonucleotides. This both prevents intra-plasmidic recombination and removes the ampicillin-resistance gene promoter.

5 The *rrn* T1 terminator was also introduced at the 3' end of the 434cI coding sequence to terminate the 434cI gene transcripts. This rho-independent terminator was chosen since its reported bi-directional termination activity would avoid any
10 disruption of 434cI expression from opposing transcripts initiated elsewhere in the vector as well as terminating 434cI transcripts.

2.4 Construction of plasmid pTP1.

15 Oligonucleotides were used to introduce the wild-type *trp* promoter sequence together with the consensus Shine-Dalgarno sequence (AGGAGGT) 5 base-pairs upstream of the 434cI gene translational start site. This spacing gives maximal translational activity of the 434cI gene. Due to the lack of
20 convenient restriction sites at the start of the 434cI gene, the *EcoRI* site 33 base-pairs inside the coding sequence was used. This necessitated the inclusion of the 5' end of the coding region in the oligonucleotide. The required sequence is 126
25 base-pairs long and thus was constructed from four overlapping oligonucleotides. Following the annealing of these oligonucleotides, the duplex 126 base-pair fragment was isolated and cloned into *EcoRI*-cleaved pUC19 vector. Following the selection
30 of transformants on media containing ampicillin and BCIG, DNA from several white colonies was sequenced using plasmid sequencing protocols. This confirmed the structure of the promoter sequence to be as expected.

2.5 Construction of pTT1.

The sequence of the rrn T1 terminator has a long G = C rich stem structure flanked by long A=T rich regions making it a strong terminator for transcripts in both orientations. Due to the inverted repeat nature of this sequence, it was inserted using four oligonucleotides thereby avoiding any problems of self-annealing within each strand. The oligonucleotides were annealed pairwise and the resultant double-stranded DNAs isolated separately and ligated to EcoRI/HindIII cleaved pTP1 DNA, prior to transformation of cells to ampicillin-resistance. The sequence of the inserted terminator structure was ascertained.

15 2.6 Construction of plasmid pTRT1

The source of the 434cI gene to be cloned behind the trp promoter was plasmid pRP42-76. Silent mutations have been introduced, using in vitro mutagenesis, to create restriction sites for KpnI and XmaIII on either side of the sequence coding for the $\alpha 3$ recognition helix. This will subsequently allow the introduction of oligonucleotides in which certain codons in the $\alpha 3$ helix have been randomly mutated. The EcoRI/Sau3A fragment (approx. 600 base-pairs) carrying the 434cI gene was isolated and cloned into EcoRI/BglII cleaved pTP1. This reforms the 434cI open reading frame exactly and the translational stop codon is retained within the Sau3A/BglII junction. Once isolated, the trpP-434cI-rrnT1 cassette was cleaved out using the BamHI sites in the polylinkers introduced at either end of the cassette and used to replace the existing 434cI gene in the pAD and pPS plasmids.

The sequence of the trp promoter used to construct pTRT1 is bound by trp repressor in the presence tryptophan to inhibit transcription. The binding site for this repressor was intentionally retained in order that expression of the 434cI gene may be controlled, if necessary, in future work. However, in order to conveniently allow the synthesis of 434 repressor, strains will be constructed in which the trpR gene has been deleted from the chromosome.

3. Selection Protocol

3.1 Selection using TONPG

A TONPG (ortho-nitrophenyl-b-thiogalactoside) selection protocol was designed which allows selection for clones in which a mutant repressor now binds to the mutant 434 operator resulting in repression of lacY expression (i.e. selection by repression of conditional inhibition).

TONPG inhibits the growth of E. coli cells that are expressing the lacY permease gene. Early work with single copy lacY+ E. coli indicated that these cells were maximally sensitive to TONPG at 500 to 1000 micrograms/ml when expressing lacY in succinate minimal medium. Mixing experiments showed that TONPG could be used to select lacY cells from a mixed lacY+/- population. These experiments were repeated with lacY+ and lacY- pAD plasmids. The TONPG selection will only work in a lac-deleted E. coli host. The preferred host is described below. Selection works better in liquid cultures, but also works on agar plates. Selection on solid medium works better with a different galactoside analogue, para-nitrophenyl-beta- thiogalactoside (TPNPG). Using TONPG in liquid cultures and TPNPG in plates, the

selection normally achieves a 6 log enrichment of lac-pAD plasmids present in the initial population.

3.2 The E.coli host

5 The bacterial host selected was such as to enable selection by TONPG. This required the ability of the host to grow in a succinate minimal medium. The particular host used in this Example was one from which the entire lac operon had been deleted, lacΔ4129. However, other suitable mutants hosts can
10 be used, for example lac⁻, lacY⁻. A suitable strain was constructed from strain W1485 (CGSC6300) derivative which was deleted for the entire lac operon, using transposon-linked P1 transduction.

3.3 Selection using TPNPG

15 To test the ability of TPNPG to select lac⁻ cells from a background of lac⁺ cells, mixing experiments with cultures of 6300lac⁺ and its Δlac4169 derivative were performed. These strains were grown to mid-log phase in the presence of 1mM IPTG, to
20 induce expression of the lactose operon. The two cultures were mixed in various proportions before plating suitable dilutions on minimal-salts-succinate plates containing 1mM IPTG and 50μg/l BCIG, both with and without TPNPG.

25 Initial experiments showed that 500μg/ml TPNPG was only able to retard the growth of lac⁺ cells, allowing the formation of small blue colonies after 48h at 28°C. However, this background was eliminated in the presence of 1mg/ml TPNPG, resulting in none of
30 the lac⁺ cells plated (up to 7×10^{-7}) being able to form blue colonies (Table V). In contrast, this TPNPG concentration did not noticeably affect the viability of the lac⁻ cells. This demonstrates the

high selective power of TPNPG against lac^+ cells, even when the lacY gene is chromosomal and thus at a low copy number. In this respect, the higher copy number and therefore increased expression level of the lac genes on the pPS plasmids should result in a greater waste of energy on TPNPG uptake, making the killing of lac^+ cells more effective.

TABLE V

ABILITY OF TPNPG TO SELECT LAC- CELLS FROM A BACKGROUND OF LAC+ CELLS				
Approx. ratio of cells plated $\text{lac}^-:\text{lac}^+$	Colonies formed/ml			
	-TONPG		+TONPG	
	White	Blue	White	Blue
1:102	1.1x10 ²	2.0x10 ⁴	1.2x10 ²	0
1:103	1.3x10 ²	2.0x10 ⁵	1.2x10 ²	0
1:104	N.D.	1.8x10 ⁶	1.1x10 ²	0
1:105	N.D.	1.5x10 ⁷	1.1x10 ²	0
1:5x10 ⁵	N.D.	7.0x10 ⁷	1.2x10 ²	0

N.D. = Not Determinable

5 The effect of TPNPG on the survival of 6300 Δ lac4169 cells carrying various pAD and pPS plasmids was tested by plating appropriate dilutions of cultures on the media as described above. The results from two experiments revealed that all plasmids resulted in the formation of both blue and white colonies in the presence of TPNPG, yet no white colonies were detected in its absence (Table VI).

TABLE VI

Plasmid carried	Colonies/ml			
	-TPNPG		+TPNPG	
	White	Blue	White	Blue
Expt#1				
-	2.8x10 ⁸	0	3.0x10 ⁸	0
pAD16	ND	3.5x10 ⁸	1.2x10 ³	2.0x10 ³
pAD17	ND	2.3x10 ⁸	1.3x10 ⁴	1.6x10 ³
pAD18	ND	2.3x10 ⁸	2.3x10 ³	2.2x10 ³
pPS1	ND	3.8x10 ⁷	2.0x10 ⁴	2.7x10 ⁷
pPS2	ND	7.2x10 ⁷	7.0x10 ²	4.8x10 ⁷
pPS3	ND	5.6x10 ⁶	2.0x10 ⁴	2.0x10 ¹
Expt#2				
-	3.1x10 ⁸	0	3.0x10 ⁸	0
pAD16	ND	2.9x10 ⁸	1.1x10 ⁴	4.2x10 ²
pAD17	ND	3.4x10 ⁸	6.7x10 ³	5.9x10 ³
pAD18	ND	2.7x10 ⁸	4.1x10 ³	2.5x10 ³
pPS1	ND	4.2x10 ⁷	8.5x10 ³	1.2x10 ⁷
pPS2	ND	8.4x10 ⁷	5.2x10 ³	1.6x10 ⁷
pPS3	ND	5.8x10 ⁷	5.7x10 ³	6.3x10 ¹

ND = Not Determinable

As already observed, all pAD and pPS plasmids give blue colonies on media containing BCIG, irrespective of the presence of 434

repressor/operator interactions. Presumably

5 therefore, the white colonies formed must result

from cells carrying plasmids which have been mutated or deleted to render the cell effectively lac^- . The relatively low frequency with which these white colonies occur (approx. 10^{-5} of cells plated) suggests that, on media lacking TPNPG, they would remain undetected amongst the majority of blue colonies. Analysis of the plasmids harboured by cells of such white colonies revealed deletions (see above).

In the presence of TPNPG the frequency of blue colonies formed by pAD-carrying strains was reduced by 10^5 to 10^6 . This represents the killing by TPNPG of the majority of the population harbouring an un-repressed lac operon. The strain carrying pPS3 was also killed to a similar extent in the presence of TPNPG, as would be expected given that it has already been shown that the 434 repressor is incapable of inhibiting transcription of the lac genes in this plasmid. However, in all cases, a residual number of blue colonies were obtained in the presence of TPNPG, at a frequency of approximately 10^{-5} of the cells plated. It is presumed that these colonies primarily represent cells harbouring lacY^- mutant plasmids, since it has already been demonstrated that the selective power of TPNPG is sufficient to kill the vast majority of the cells plated, given that they all remain lac^+ . Since previous experiments have not indicated a high mutation frequency for the chromosomally borne lacY gene, it is presumed that intra-plasmidic recombination is responsible for the apparently high number of mutants. Previous work has indicated that these plasmids are prone to

instability in rec^+ strains and that a 6300 Δ lac4169 $recA56$ strain, which could be used to prevent such recombination, is inviable on TPNPG.

5 However, in sharp contrast to the other
strains, the vast majority of cells containing pPS1
or pPS2 survive in the presence of TPNPG, the
number of blue colonies being reduced by only 2-5
fold, this reduction being least for pPS1. This
clearly correlates with the emphatic reduction in
10 β -galactosidase activity, and therefore also
presumably lacY expression, already demonstrated
for these plasmids. Therefore, the important
conclusion can be drawn that the interaction
between 434 repressor and its cognate operator is
15 able to reduce lac gene expression sufficiently to
allow the majority of cells to survive the
selective procedure.

4. Selection of altered specificity repressors

4(a) Selection of altered 434 repressor

20 A 434 gene which had been altered to
facilitate random mutagenesis of the 434 repressor
binding domain through insertion of random
oligonucleotides has been described (Wharton and
Ptashane, Nature 316, 601-605). The 434 repressor
25 gene has been mutagenized to introduce KpnI and
NotI restriction enzyme cleavage sites on either
side of the DNA recognition helix, whilst
conserving the native amino acid sequence. Batches
of oligonucleotides have been synthesised with the
30 correct cohesive ends and containing varying
frequencies of mutations randomly distributed
throughout the DNA recognition alpha helix. These
oligo mixtures have been cloned between the KpnI
and the NotI cohesive ends of the modified 434

repressor gene. Alternatively the 'dirty oligo' approach has been used for the generation of mixed oligo with base substitutions at appropriate positions of the DNA binding domain.

5 4(b) Selection of altered 434 repressor
 recognising pseudo-operator found in plant genes

A naturally occurring pseudo-operator was used for the selection of altered repressor. The target for this work was the GPAL2 gene from French bean, the
10 chlorophyll a/b binding protein gene from maize, and others. We have identified by computer analysis, that several potential 434 pseudo-operators are located in the region of the GPAL2 gene. These regions of the GPAL2 promoter
15 were used to select an altered specificity 434 repressor. The 'pseudo-opertor' sequences were inserted into the -10 to -35 region of the lac promoter driving the lacZ/lacY genes. Dirty oligo's were inserted into the 434 repressor gene
20 and mixtures were transformed into E.coli 6300. The selection protocol was applied and colonies isolated. Using microbiological and molelcular techniques we have demonstrated that mutant repressors can be selected for. The
25 characterisation of the repressor gene has been done by DNA sequence analysis, and binding studies to determine the strength of the repressor binding.

In summary, then, the present invention provides a selection system comprising preferably
30 of bacterial strains and plasmids, and a sensitive suicide substrate selection protocol. This selection system can be used to select altered specificty repressors. Implied in this invention is the provision of controlling gene expression in

organisms by said altered- specificity repressors. The only requirement for this method of control of gene expression are the DNA sequence of the target gene, the identification of 'pseudo-operators' being a DNA sequence that resembles the normal operator sequence and a selection system which permits the selection of repressors capable of binding to said 'pseudo-operators'.

III. MALE FLOWER SPECIFIC GENE SEQUENCES

This module contains male flower specific DNA sequences comprising the polynucleotides shown in Figures 17, 18, and 19 herewith, which are specifically expressed in male flower tissue.

Plasmid pMS10 in an Escherichia coli strain R1 host, containing the gene sequence shown in Figure 17 herewith, has been deposited with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40098.

Plasmid pMS14 in an Escherichia coli strain DH5 α host, containing the gene control sequence shown in Figure 18 herewith, has been deposited with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40099.

Plasmid pMS18 in an Escherichia coli strain R1 host, containing the gene control sequence shown in Figure 19 herewith, has been deposited with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40100.

The isolation and characterisation of these cDNA sequences and the utilisation of these cDNA sequences as molecular probes to identify and isolate the corresponding genomic sequences will

now be described.

The clones carrying the genomic sequences and the preparation of a promoter cassette from one of the clones illustrated using an approach and techniques which may be equally applied to any of the the clones. Furthermore the preparation of a promoter fusion to a reporter gene and the transformation of this construct into a test species is described.

Unless stated otherwise, all nucleic acid manipulations are done by standard procedures described in Sambrook, Fritsch and Maniatis, "Molecular Cloning: A Laboratory Manual", Second Edition 1989.

EXAMPLE 1

1. Isolation and Characterisation of Male Flower Specific cDNA from Maize

To clone cDNAs to genes which are expressed in the male flowers of maize we constructed two cDNA libraries. In maize, the male flowers are born in the tassel which terminates the main stem. Library 1 was prepared from poly [A] RNA from whole maize tassels bearing early meiotic anthers (most meiocytes in early meiotic prophase) and library 2 from poly [A]⁺ RNA from whole tassels bearing late meiotic anthers (predominantly diad and early tetrad stages). Figure 14 reviews the library screening procedure used and this yielded five unique early meiotic MFS cDNAs and one unique late meiotic cDNA. Clone PMS3, a partial cDNA of 120 base pairs, isolated by the differential screening process, was subsequently used as a hybridisation probe to isolate the corresponding pending near full-length clone, PMS18.

Table VII below summarises some of the features of each of these cDNA clones. Expression of the mRNAs of the five MFS cDNAs isolated from the early meiotic library is detected in RNA isolated from both early and late meiotic tassel samples. The mRNAs corresponding to these cDNAs are not wholly specific to male flowers and are detected at considerably lower levels in leaves (pMS10 and pMS18) or in leaves, cobs and roots (pMS1, pMS2 and pMS4) Table VII. In contrast pMS14 mRNA is found only in late meiotic RNA and is not detected in leaves, cobs or roots (Table VII).

TABLE VII						
	pMS1	pMS2	pMS4	pMS10	pMS14	pMS18
Library ¹	1	1	1	1	2	1
Insert size ²	750	500	720	1350	620	940
mRNA size ³	900	950	850	1600	900	1100
Organ specificity ⁴	+	+	+	++	+++	++
Expression window ⁵	E/L	E/L	E/L	E/L	L	E/L

Table Legend

- 1 Isolated from cDNA library 1 (early meiotic)
or library 2 (late meiotic).
- 2 Approximate size in base pairs.
- 5 3 Approximate size in nucleotides.
- 4 + = expressed in tassels and at much lower
levels in leaves, cobs and roots.
++ = expressed in tassels only and at much
lower levels in leaves.
- 10 +++ = expressed in tassels only.
- 5 E/L = mRNA present in RNA from both early and
late meiotic tassels.
L = mRNA present only in RNA from late meiotic
tassels.
- 15 We have examined expression of the genes
corresponding to these cDNAs during tassel
development using dot blot hybridisations (Figure
15). The dot blot analysis was generated by
binding total; RNA to nitrocellulose followed by
- 20 hybridisation to radiolabelled pMS cDNAs. All
filters were exposed to film for 48 hours at -70°C
except pMS10 which was exposed for 168 hours. The
tassel lengths in each sample were as follows: A ≥
2cm; B=2-5cm; C=5-10cm; D=10-15cm; E= 15-20cm;
- 25 F=20-30cm; and G=20-30cm. The solid bars in Figure
15 show the developmental stage relative to
microsporogenesis in each of the samples: PM =
premeiosis; M = meiosis; IP = immature pollen; and
MP = mature pollen.
- 30 The early meiotic mRNAs (pMS1, 2, 4, 10 and
18) accumulate very early in development in tassels
less than 2 cm in length. We have not analysed
expression in floral meristems prior to this stage.
These mRNAs persist through the meiotic anther

stages and then decline as pollen grains mature. In contrast the late meiotic mRNA of pMS14 is not detected in tassels less than 5 cm in length, but increases dramatically as the sporogenous cells of the anther enter meiosis (Figure 15). As with the early meiotic mRNAs, pMS14 mRNA declines abruptly as mature pollen accumulates in the anthers (Figure 15).

These data show that different temporal controls of gene expression occur during development of male flowers in maize. The controls which programme accumulation of the early meiotic mRNAs are probably very similar but contrast markedly with those regulating appearance and accumulation of the late meiotic mRNA, pMS14. Both the early and late meiotic mRNAs are involved with developmental processes which occur prior to the accumulation of mature pollen grains. They are clearly not involved with the later stages of anther development such as dehiscence nor are they mRNAs which accumulate in mature pollen.

The technique of in situ hybridisation has been used to determine the tissue localisation of MFs mRNAs in male flowers of maize. The techniques used are described in Wright and Greenland (1990; SEB Seminar Series, vol 43 ed by N Harris and D Wilkman. Cambridge University Press, Cambridge; in the Press). The data shown is that for pMS14 mRNA.

Figure 16 A,B shows in situ hybridisation with pMS14 antisense RNA probes. Sense and antisense probes more prepared by sub cloning a 300 basic pair fragment of pMS14 into the vector, pBS, followed by preparation of radiolabelled T3 and T7 polymerise transcripts utilising methods suggested

by the supplier of the vector (Stratagene, Trade Mark). These hybridisations show that pMS14 mRNA is located in the tapetal cell layer surrounding the developing microspores. Hybridisation of the pMS14 antisense probe does not occur to any other cells in the section. Likewise the pMS14 sense probe does not show any specific hybridisation (Figure 16C). These sections were made from 15-20 cm maize tassels at a stage when the level of pMS14 mRNA is at a maximum (Figure 15). In these sections and in those from subsequent experiments hybridisation occurs to the tapetum of the anthers in one floret but not the other. In Figure 16 A,B the tapetal layers which contain pMS14 mRNA surround late meiotic microspores at the tetrad stage whilst the tapetal layers not containing pMS14 mRNA surround sporogenous cells which have not undergone meiosis. It is a feature of maize that the sets of anthers within the individual florets of the spikelet do not develop co-ordinately. Thus in situ hybridisation shows that accumulation of pMS14 mRNA is tissue-specific and confirm data obtained from dot blot analysis (Figure 15) that expression of Pms14 mRNA is stage specific as it is first detected in tapetum surrounding meiotic cells.

EXAMPLE 2

Determination of DNA sequence of pMS10

DNA from cDNA clone, pMS10, for sequence analysis by subcloning into M13mp18 using standard procedures. The nucleotide sequences of the subclones were determined by the dideoxy method using standard procedures. In addition a Sequence (Trade Mark) method was used utilising methods

described by the suppliers. Regions of the clones were sequenced by priming with synthetic oligonucleotides synthesised from sequence obtained from previous gel readings. Oligonucleotide concentrations used for priming were identical to those used with universal primers.

MFS, Clone pMS10 full length cDNA of 1353 base pairs. The complete nucleotide sequence and the predicted amino acid sequence are shown in Figure 17. The sequence contains an open reading frame of 1022 nucleotides encoding a polypeptide of 341 amino acids with a deduced molecular weight of 37371 kd the polypeptide is rich in glycine residues. The open reading frame is flanked by 5' and 3' non-translated regions of 129 and 201 bases respectively.

EXAMPLE 3

Determination of DNA sequence of pMS14

Procedure of determining nucleotide sequence as described in Example 2.

Clone pMS14 is an in complete cDNA of 581 base pairs the complete nucleotide sequence and deduced amino acid sequence are shown in Figure 18. The sequence contains an open reading frame which extends from nucleotide 1 to 278 encoding a partial polypeptide of 127 amino acids. The polypeptide is particularly rich in alanine and arginine residues. The open reading frame is flanked by 3' non-coding region 203 nucleotides. A consensus processing and polyadenylation signal hexanucleotide, AATAAA occurs at position 548.

EXAMPLE 4

Determination of DNA sequence of pMS18

Procedure for determining nucleotide sequence

as described in Example 2.

Clone pMS18 is a near full-length cDNA of 933 bases. The complete nucleotide sequence and deduced amino acid sequence is shown in Figure 19. pMS18 lacks 28 nucleotides at its 3' terminus. The missing nucleotides are present in clone pMJ3 which overlaps the sequence of pMS18 by a further 91 nucleotides. pMS3 was the original clone isolated by differential screening of cDNA inbranes and was subsequently used as a hybridisation probe to isolate pMS18. pMS18 contains an open reading frame extending from nucleotide 151 to 813 and encodes a polypeptide of 221 amino acids with a deduced molecular weight of 25 kilodartons. The polypeptide is particularly rich in arginime residues. The open reading is flanked by 5' and 3' non-coding regions of 150 and 120 nucleotides respectively.

EXAMPLE 5

Isolation of genomic clones corresponding to pMS10

Genomic DNA clones carrying genes corresponding to the cDNA, pMS10 were isolated from an EMBL 3 phase library of partial Mb01 fragments of maize DNA. The library was screened using radiolabelled "long-mer" probes synthesised in an in vitro labelling system. This system comprised, 50 mg of a synthetic 100 base oligonucleotide (base position 452-551 at pMS10; Figure 17). 500 mg of a synthetic primer oligonucleotide, sequence - TAGTTTCCT-CGGTAG and which will base pair with the 3' end of the long olionucleotide, one or two radiolabelled oligonucleotides (usually ³²PdCTP and/or ³²P-dGTP) and 5-10 units of the Klenow

fragment of DNA polymerase 1. The reactions were performed at 37°C for 30 minutes in a buffer identical to that used for the "random-priming" method of DNA labelling except that the random hexanucleotides were omitted. Five million phase clones immobilised on nylon "Hybaid" (Trade Mark) filters were hybridised at 65°C with these probes using prehybridisation and hybridisation buffers suggested by the suppliers of the filters (Amersham International). Filters were washed on 3 x SSC, 0.1 % SDS at 65°C using these procedures 50-60 EMBL3 phage clones containing either complete or partial regions of a pMS10 gene were obtained. The DNA from three EMBL3 phage clones 10/CT8-1, 10/CT8-3 and 10/CT25-3 which combined complete pMS10 genes was prepared and analysed by restriction enzyme digests. Each of these clones was shown to contain a common 9Kb EcoRI fragment which extends from the third intron of the pMS10 gene into the 5' non-coding and promoter regions of the gene. A partial restriction map of the 9 Kb EcoRI fragment is shown in Figure 20.

EXAMPLE 6

Isolation of genomic clones corresponding to pMS14

To isolate genomic DNA clones carrying genes corresponding to the cDNA, pMS14 two approaches were taken. In the first approach the method shown in Example 5 was adopted except the 5 million phage clones were screened with the complete cDNA sequence and the wash stringencies after hybridisation procedure yielded two positive clones 14/CTA and 14/CTD. In the second approach a 12 Kb EcoRI cut fraction of maize genomic DNA, shown by Southern Blotting to carry the pMS14 gene, was

ligated into EcoRI cut λ phage EMBL4 DNA to produce a library of cloned 17 Kb DNA fragments. Roughly 200,000 clones were screened as described above, and two positive clones, 14/17m and 14/17R which combined a 17 Kb EcoRI fragment which hybridized to pMS14, were isolated. On further analysis the two positive clones isolated from the partial MboI/EMBL3 library were found to contain an internal 17 Kb fragment. A partial restriction map of this 17 Kb EcoRI fragment, common to all the clones, is shown in Figure 21.

EXAMPLE 7

Isolation of genomic clones corresponding to pMS18

To isolate genomic DNA clones carrying genes corresponding to the cDNA pMS18, the procedure described in Example 5 was adopted. Five million EMBL3 phage clones were hybridized to a "long-mer" probe derived from the sequence of pMS18, position 133-222). The sequence of the 3' complementary oligonucleotide was a 5'-GCCTCGGCGGTCGAC-3'. Two clones, 18/CT3 and 18/CT23, carrying the pMS18 gene were isolated from this screen. Restriction mapping of these clones showed that they both contained a 4.5 Kb BamHI-SalI fragment comprising the 5' region of the coding sequence of pMS18 and approximately 4 Kb of the promoter and upstream region of the gene. A partial restriction map of clone 18/CT3 is shown in Figure 22.

EXAMPLE 8

Construction of a promoter cassette derived from 10/CT8-3

The following subclones from the λ EMBL3 clone 10/CT8-3 were made. The 4.5 Kb PstI-EcoRI fragment was cloned into pUC18 to give pMS10-2. The 2.7 Kb

XbaI-EcoRI fragment was cloned into pUC 18 to give pMS10-3. The 1.6 Kb HindIII to XbaI fragment was cloned into pUC18 to give pMS10-4.

5 The polymerase chain reaction (PCR) was used
to amplify a 930 bp fragment from pMS10-3. The
primers used for the PCR reaction were as follows.
Primer pUC/2 is homologous to pUC sequence flanking
the polylinker site. Primer 10/9 is complementary
10 to the sequence of pMS10 from position 106-129
except that it contains an additional thymidine
residue between bases 123 and 124. The sequence of
these primers is:

pUC/2 5' CGACGTTGTAAAACGACGGCCAGT-3'

10/9 5' AGTCGGATCCCGCCCCGCGCAGCCG-3'

15 Following amplification in the PCR reaction a
DNA fragment is produced in which the flanking XbaI
site and the sequence identical to that present in
the corresponding region of clone 10/CT8-3 up to
the base immediately prior to the translation
20 initiator are faithfully reproduced except that a
novel BamHI site is introduced by the introduction
of the thymidine residue. This 930 bp fragment was
gel purified, and digested with XbaI and BamHI. It
was then cloned into pMS10-4 which had been
25 previously digested with XbaI and BamHI to yield
clone pMS10-5. In pMS10-5 the sequences required
for promoter activity associated with the MS10 gene
are reacted and modified such that the promoter can
now be fused to any gene via the BamHI site which
30 occurs immediately prior to the translation start
point. That these and no other modifications had
occurred was confirmed by sequence analysis.

EXAMPLE 9Construction of a promoter fusion between MS10 gene and the glucuronidase reporter gene

5 The 1830 bp HindIII to BamHI fragment from pMS10-5 (Figure 38) was ligated into pTAK1, previously cut with HmdIII and Bam HI. pTAK1 is based on the binary plant transformation vector Bin 19 (Bevan, 1984; Nucleic Acids Research 12, 8711) and carries the glucuronidase (GUS) reporter gene and Nos 3' terminator (Figure 24). The resulting
10 plasmid was termed pMS10-6GUS and makes a transcriptional gene fusion between the promoter of the MS10 gene and the GUS reporter gene.

EXAMPLE 10

15 Transformation of tobacco plants with MS10 promoter gene constructs

The recombinant vector pMS10-6GUS as mobilised from E. Coli (TG-2) onto Agrobacterium tumefaciens (LBA4404) in a triparental mating on L-plates with
20 E Coli (HB101) harbouring pRK2013. Transconjugants were selected on minimal medium containing kanamycin ($50\mu\text{g}/\text{cm}^3$) and streptomycin ($500\mu\text{g}/\text{cm}^3$).

L-Broth (5 cm^3) containing kanamycin at $50\text{ g}/\text{cm}^3$ was inoculated with a single Agrobacterium
25 colony. The culture was grown overnight at 30°C with shaking at 150 rpm. This culture ($500\mu\text{l}$) was inoculated into L-Broth containing kanamycin ($50\mu\text{ g}/\text{cm}^3$) and grown as before. Immediately before use the Agrobacteria were pelleted by
30 spinning at 3000 rpm for 5 minutes and suspended in an equal volume of liquid Murashige and Skoog (MS) medium.

Feeder plates were prepared in 9 cm diameter petri dishes as follows. Solid MS medium

supplemented with 6-benzyl-aminopurine (6-BAP) (1 mg/1) and 1-naphthaleneacetic acid (NAA) (0.1 mg/1) was overlaid with Nicotiana tabacum var Samsun suspension culture (1 cm³). One 9 cm and one 7cm
5 filter paper discs were placed on the surface.

Whole leaves from tissue culture grown plants were placed in the feeder plates. The plates were sealed with "Nescofilm" (Trade Mark) and incubated overnight in a plant growth room (26°C under bright
10 fluorescent light).

Leaves from the feeder plates were placed in Agrobacteria suspension in 12 cm diameter petri dishes and cut into 1- 1.5 cm² sections. After 20 minutes the leaf pieces were returned to the feeder
15 plates which were sealed and replaced in the growth room. After 48 hours incubation in the growth room the plant material was transferred to MS medium supplemented with 6-BAP (1 mg/1), NAA (0.1 mg/1), carbenicillin (500 µg/cm³) and kanamycin (100
20 µg/cm³), in petri dishes. The petri dishes were sealed and returned to the growth room.

Beginning three weeks after inoculation with Agrobacterium, shoots were removed from the explants and placed on MS medium supplemented with
25 carbenicillin (200 µg/cm³) and kanamycin (100 µg/cm³) for rooting. Transformed plants rooted 1-2 weeks after transfer.

Following rooting, transformed plants were transferred to pots containing soil and grown in
30 the glasshouse. Roughly one month after transfer the plants flowered.

The anthers of the tobacco plants containing the pMS10-6GUS construct were sprayed for GUS activity using standard procedures.

IV. DISRUPTER PROTEIN GENE

This module contains a gene, expressible in plants, to inhibit mitochondrial function, hence disrupting full expression of a selected plant characteristic.

Accordingly we provide a method of inhibiting gene expression in a target plant tissue comprising stably transforming a plant cell of a type from which a whole plant may be regenerated with a gene construct carrying a tissue-specific or a development-specific promoter which operates in the cells of the target plant tissue and a disrupter gene encoding a protein which is capable, when expressed, of inhibiting respiration in the cells of the said target tissue resulting in death of the cells.

Preferably the disrupter gene is selected from:

(a) The mammalian uncoupling protein (UCP) cloned from mammalian (usually rat) brown adipose tissue.

(b) A mutated form of the gene for the β -subunit of F_1 -ATPase which has sequences added or deleted such that these changes result in the retention of the ability to assemble with other subunits but interfere with function as an ATP synthase. The ability of these altered subunits to assemble correctly will be important as the required phenotypic effect of their expression will depend on their competition with wild-type subunits for binding sites in the enzyme complex. Thus complexes containing non-functional subunits will only be weakly active and mitochondria harbouring these complexes will be non-functional.

(c) A mutated, synthetic form of the oli 1 gene

encoding subunit 9 of the F_0 -ATPase. Mutations created as described at (b) above.

(d) A mutated form of a mitochondrial transit pre-sequence which malfunctions during transfer resulting, probably by blocking of receptor sites, in the disruption of protein transport to mitochondria.

(e) Gene constructs involving a fusion between the β -subunit gene from yeast and the β -galactosidase gene from E. coli, resulting in expression of a disrupting fusion protein.

Preferably the promoter is a tapetum-specific promoter or a pollen-specific promoter, so that on expression of the said disrupter protein therein the regenerated plant is in male sterile. More preferably the said tapetum-specific promoter has the sequence shown in Figure 17, 18, or 19 of the accompanying drawings.

The isolation and characterisation of these gene control sequences of this invention are described in section III above.

This module, therefore, provides a method of preventing or inhibiting growth and development of plant cells based on gene constructs which inhibit respiratory function. The technique has wide application in a number of crops where inhibition of particular cells or tissue is required.

Of particular interest is the inhibition of male fertility in maize for the production of F1 hybrids in situ. The concept of inhibition of mitochondrial function as a mechanism for male sterility arises from some previous research on T-type cytoplasmic male sterility in maize (cms-T) which has shown an association between the male

sterile phenotype and mitochondrial dysfunction. Although a direct causal relationship has yet to be established between mitochondrial dysfunction and cms-T, an increasing body of evidence suggests that
5 fully functional mitochondria, particularly in the tapetal cells, are essential. This is particularly critical during microsporogenesis since the metabolic demands placed on the tapetal cells results in a 40-fold increase in mitochondrial
10 number.

Thus we provide a number of negative mutations which act upon mitochondria to uncouple oxidative phosphorylation. When specifically expressed in maize anther tissue these mutations will result in
15 a male sterile phenotype.

The proposed disrupter protein, UCP, is instrumental in the thermogenesis of mammalian brown adipose tissue and exists as a dimer in the mitochondrial inner membrane forming a proton
20 channel and thus uncoupling oxidative phosphorylation by dissipation of the proton electrochemical potential differences across the membrane.

An alternative is the use of chimeric gene constructs in which domains are swapped, creating non-functional proteins. The target proteins here are the β -subunit of F_1 -ATPase and subunit 9 of the F_0 -ATPase. During assembly of functional ATPase complexes, the altered chimeric subunits will
25 complete for binding sites normally occupied by the naturally occurring subunits, particularly when the chimeras are over expressed compared with the endogenous genes. Mitochondrial function will be
30 disrupted since F_1 and F_0 ATPase's assembled with

altered subunits are likely to be weakly active or non-functional.

5 The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection
10 using Agrobacterium tumefaciens or its Ti plasmid, electroporation, microinjection of plant cells and protoplasts, microprojectile transformation and pollen tube transformation, to mention but a few. Reference may be made to the literature for full
15 details of the known methods.

The development and testing of these gene constructs as disrupters of mitochondrial function in the unicellular organism, yeast, will now be described. A mechanism by which these gene
20 constructs may be used to inhibit plant cell growth and differentiation in transformed plants will also be described. The object of these procedures is to use yeast as a model system for the identification and optimisation of gene constructs for expressing
25 proteins which disrupt mitochondrial function. Plant cells will then be transformed with the selected constructs and whole plants regenerated therefrom.

Specific embodiments of the module will now be
30 illustrated by the following Example.

EXAMPLE

It was known from reports in the literature that the rat UCP gene inserted in the yeast/E. coli shuttle vector gave only low levels of expression

of UCP. The yeast was Saccharomyces cerevisiae strain YM147 and the UCP gene was available on plasmid pCGS110-UCP.

5 Given the lack of useful expression levels with the wild type gene, modification of the rat UCP gene using site directed mutagenesis was carried out. the following modifications were made:

- 10 1. Introduction of a BamHI site seven nucleotides 5' to the AUG methionine initiation codon;
2. Modification of the sequence around the AUG methionine initiation codon to conform to the yeast consensus sequence ATAATG;
- 15 3. Deletion of an internal BamHI site; and,
4. Introduction of a BamHI site one nucleotide 3' to the TAG termination codon.

These modifications result in the deletion of the untranslated 5' and 3' rat UCP sequences as well as the introduction of a yeast consensus
20 sequence at the methionine initiation codon.

The 1.9 kb EcoRI/PstI fragment from the plasmid pCGS110-UCP (a map of the plasmid is shown in Figure 26 and the mRNA sequence of the UCP gene is shown in Figure 27) carrying the GAL10 promoter
25 region and the rat UCP cDNA was cloned into the EcoRI/PstI sites of M13mp19 DNA. Sequencing of the resultant construct was carried out to ensure the correct structure.

30 Site directed mutagenesis was carried out according to the directions given in the Amersham (Trade Mark) mutagenesis kit using three different oligonucleotides as follows:

UCP-1 wild type CTCTGCCCTCCGAGCCAAGATGGTGAGTT
mutant CTCTGCCCTCCGATCC(ATAATG)GTGAGTT
UCP-2 wild type TGCGACTCGGATCCTGGAACG
mutant TGCGACTCGGTTCCCTGGAACG
5 UCP-3 wild type ACCACATAGGCGACTTGGAG
mutant ACCACATAGGATCCGACTTGGAG

Oligonucleotide UCP-1 was used to introduce the yeast consensus sequence (bracketed) which occurs around the methionine initiation codon, as well as the introduction of the BamHI cleavage site (underlined).

Oligonucleotide UCP-2 was used to delete an internal BamHI site (underlined).

15 Oligonucleotide UCP-3 was used to introduce a BamHI site immediately after the TAG stop codon (underlined).

These three mutations allowed the isolation of the entire UCP coding sequence on a 0.93 kb BamHI fragment.

After selection of mutant clones the modified DNA was digested with BamHI. Three clones from twenty selected gave inserts of 0.93 kb upon digestion with BamHI.

25 Sequencing of the clones UCPS1 and UCPS4 revealed that the UCP gene had been correctly modified with no unwanted changes present. The UCP gene was then transferred to the yeast expression plasmid pKV49 which allows expression of foreign genes in S. cerevisiae under the control of the strong PGK promoter and the GAL1-10 UAS allowing induction/repression of the foreign gene according to whether or not galactose is present in the growth medium. The 0.93 kb BamHI fragment

containing the modified UCP gene was cloned into pKV49 at the BglII restriction site, resulting in the construct pKV49-UCP.

TRANSFORMATION OF YEAST WITH pKV49-UCP CONSTRUCT

5 a) Development Of Suitable Yeast Strain

For a recipient for the pKV49-UCP construct we needed a yeast strain carrying the appropriate markers for transformation and allowing induction of gene expression from the GAL1-10 UAS while being
10 unable to utilise glactose as a carbon and energy source (GAL1, GAL2). Such strains were generated by mating yeast strains YM147 and SF747. After selection of diploids on minimal plates containing uracil, the colonies were transferred to
15 sporulating media. The resulting spores were grown on YDP plates prior to the resulting yeast colonies being characterised (Figure 28). Two new yeast strains BET9 (ura3, trp11, leu2, his3, gal1) and BE27 (ura3, trp1, leu2, gal1) were isolated, both
20 of which are suitable for transformation with Pkv49 based constructs.

b) Yeast Transformation

Yeast strains BET9 and BE27 were transformed with pKV9 and pKV49-UCP DNA; transformants were
25 selected using the appropriate auxotrophic selection (leu) and checked by plasmid isolation followed by restriction mapping. Single colonies from each of the four different transformants BET9/pKV49, BET9/pKV49-UCP, BE27/pKV49 and
30 BE27/pKV49-UCP) were resuspended in sterile water prior to being spotted onto plating media containing a variety of carbon sources (Figure 29) both in the presence and absence of galactose. Results from these plate tests (Figure 29)

indicated that on a few of the carbon sources used, the presence of both galactose and the pKV49-UCP construct resulted in poorer growth of the resulting yeast colonies. The greater effect on retardation of growth was observed with the glycerol/casamino (gly/cas) medium containing galactose for both pKV49-UCP transformants. Transformants either lacking the UCP gene or induced by galactose grew at the same rate as the untransformed BET9 and BE27 strains.

GROWTH CURVE ANALYSIS

As plating tests had indicated poor growth of pKV49-UCP transformants on gly/cas medium in the presence of galactose, growth curve analysis in liquid culture was carried out to determine more accurately the magnitude of the growth defect. The results in Figure 30 substitute the results of plating tests and indicate that neither the presence of pKV49-UCP DNA or galactose alone is sufficient to have any effect on the yeast cell growth rates, while the presence of both severely retards growth. As our initial results using the yeast strain YM147 transformed with the construct pCGS110-UCP had not shown any significant growth defect on any of the tested carbon sources in the presence of galactose, it would appear that the modification of the UCP gene and/or the use of a different vector (pKV49) have resulted in an observable growth defect.

ANALYSIS OF UCP EXPRESSION

As the growth curve analysis had indicated no detectable differences between the BE27 and BET9 transformants (Figure 30), it was decided only to use the BET9 transformants in subsequent

experiments. Repeat growth analysis on gly/cas medium both in the presence and absence of galactose was carried out with the BET9 transformants. Cultures were allowed to grow for 47 hours to ensure that the same growth curve characteristics observed previously (Figure 31) were repeated. Cells were then harvested, total cell proteins were isolated and fractionated (in duplicate) by SDS-PAGE on a 10% polyacrylamide gel. One set of fractionated proteins were stained with Coomassie Blue to ensure equal loading of the proteins while the other set were transferred to nylon membrane and subjected to Western blot analysis using the rat UCP antibody. The Western blot showed two main features:

- 1) The comparative level of UCP expression between the BET9/pKV49-UCP transformant and the VY147/pCGS110-UCP transformant reveals that the UCP expression has increased approximately 50-100 fold as a consequence of our modifications.
- 2) The yeast transformant which exhibits defective growth when grown on gly/cas medium in the presence of galactose also expresses substantial amounts of UCP.

It can be concluded from these results that the modification of the UCP gene and/or its subsequent cloning into the pKV49 vector has resulted in the increased level of UCP expression relative to the levels initially detected with the pCGS110-UCP construct. Growth curve analysis indicates that the expression of UCP has an effect on the growth rates of yeast cells grown under certain conditions. As yet we have not been able

to identify the specific effect that the increased levels of UCP expression have on yeast cell growth rates but preliminary results implicate a mitochondrial defect.

5 Growth curve analysis carried out in the raffinose medium (a fermentable carbon source which should not affect Gal regulation) of the BET9 transformants grown in the presence or absence of galactose indicate that the presence of both the
10 UCP gene and galactose has no effect on growth rates (Figure 32). Western blot analysis of the proteins isolated from cells harvested during these growth curves reveals levels of UCP expression similar to those found in cells grown in gly/cas
15 medium in the presence of galactose.

 The UCP detected in BET9/pKV49-UCP transformants grown without added galactose is probably due to galactose residues released into the medium by hydrolysis of raffinose, possibly
20 during the autoclaving. These observations indicate that the presence of UCP in yeast cells grown on a fermentable carbon source (no requirement for oxidative phosphorylation) has no effect on cell growth rates, while cells growing on
25 the gly/cas medium (a non-fermentable carbon source) expressing UCP exhibit defective growth.

LOCATION OF UCP IN YEAST CELLS

 Rat UCP is a major component of the mitochondrial inner membrane of brown adipose
30 tissue. Unlike many other polypeptides found in the inner membrane it does not contain a cleavable signal sequence, the targeting information being encoded internally within the amino acid sequence of the protein. As our results indicate that the

expressed UCP has an effect on the rate of yeast cell growth then it is important to determine the precise location of the protein expressed in yeast cells. Initial Western blot analysis of total
5 mitochondrial proteins shows the UCP expressed by the pKV49-UCP transformant to be located in the mitochondrial fraction.

Subsequent mitochondrial fractionation revealed that the majority of the UCP is located in
10 the inner membrane fraction of yeast mitochondria. Although some of the UCP appears to be located in the inner-membrane space, this observation is most likely due to contamination of this fraction with some of the inner membrane fraction. Similar
15 results have been obtained with the location of the β -subunit of the F_1 -ATPase complex from yeast cell mitochondria. The β -subunit which is a component of the inner membrane is also detected in our inter-membrane space preparations. However, these
20 results do show that the targeting information within the rat UCP is sufficient to target the UCP to the inner membrane of yeast mitochondria where it could function as an uncoupler protein.

UCP TRANSCRIPT ANALYSIS

25 RNA has been isolated from many of the growth curve experiments described previously. We are currently carrying out Northern blot analysis in order to determine whether the patterns of UCP expression are reflected by the UCP transcript
30 signals

EFFECT OF COPY NUMBER ON UCP EXPRESSION

The transformation of yeast cells with shuttle vectors containing the origin of replication from the yeast 2 μ m circle, such as pKV49-UCP, results

in these plasmids being present at approximately 40-50 copies per cell. Consequently any foreign gene carried by the plasmid will be present at the same relatively high copy number which may result in the expression of the foreign protein at a higher level than would be seen for a gene present at a low copy number. We have therefore attempted to lower the copy number of the UCP gene by integrating it into the yeast chromosome at a single site resulting in a genetically stable, single-copy transformant. The vector YIp5 (Figure 33) is an integrating yeast vector carrying the ura3 gene; it is unable to replicate autonomously in yeast.

The 1.8 kb EcoRI/SalI fragment from pKV49-UCP containing the rat UCP gene along with the PGK promoter and GAL UAS was cloned into the EcoRI/SAL I sites of YIp5 DNA. The resultant plasmid UIP-UCP (Figure 33) was checked by restriction enzyme mapping to ensure the UCP gene was correctly inserted. The YIP-UCP plasmid was cut with the restriction enzyme EcoRV (which cuts in the middle of the URA3 gene (Figure 33) and the linearised YIP/UCP DNA was used to transform the yeast cell lines BET9 and BE27. Transformants were initially screened on minimal plates by selecting for uracil prototrophy and after 7-10 days two transformants from each cell line were streaked out onto YPD plates (non-selective). The transformants were then subjected to four consecutive periods of growth on non-selective medium. One hundred colonies from each of the original four transformants were then replica plated onto both non-selective (YDP) and selective media (minimal

plants + ura). All colonies grew on the selective media indicating that the URA3 gene, which is genetically linked to the UCP gene (Figure 33), had been integrated to the yeast cell chromosome.

5 Chromosomal DNA was isolated from each of the four transformants, digested with the restriction enzyme EcoRI and fractionated on a 0.08% agarose gel. Southern blot analysis using a labelled UCP probe indicated that the UCP gene is present in the yeast
10 chromosome of all four transformants. Western blot analysis using the rat UCP antibody will show the level of UCP expression in these transformants. Growth curve analysis of these transformants grown in the presence of galactose shows that they may
15 have growth inhibition consistent with a mitochondrial defect.

EXAMPLE 3

MODIFICATION OF THE β -SUBUNIT OF F_1 ATPASE

The second approach we have taken to
20 introducing mutations affecting mitochondrial function is the directed modification of functional mitochondrial proteins which when expressed in yeast might be expected to interfere with the generation of ATP. The protein chosen for this
25 approach is the β -subunit of the F_1 -ATPase complex. The DNA sequence of the yeast β -subunit gene is known and the gene has been independently cloned and sequenced in our laboratory (918).

The F_1 ATPase portion of ATP synthase catalyses
30 the terminal step of oxidative phosphorylation. F_1 is an assembly of five different polypeptides designated α , β , γ , δ and ϵ . Experiments carried out by Parsonage et al on modification of the β -subunit of F_1 -ATPase from E. coli identified

specific amino acid residues of the β -subunit that appear to be very important for catalysis of both ATP synthesis and hydrolysis. Two mutations in particular were shown to result in greatly impaired catalysis without causing major structural perturbation of the F_1 -ATPase. One of these mutations resulted from changing the strongly conserved lysine residue occurring in the catalytic nucleotide-binding domain at position 155 to a glutamine residue while the other mutation resulted from changing the methionine residue at position 209 to a leucine residue. Both of these mutations have been reposed to exert their effect by the prevention of conformational changes required from the catalytic cooperativity in the F_1 complex.

As the assembly of these mutated β -subunit proteins into the F_1 -ATPase is not affected, then it was felt that similar mutants of the β -subunit in yeast might compete for assembly into F_1 -ATPase. It was thought that the result of having both wild-type and mutated β -subunits in the same F_1 -ATPase would perhaps result in impaired catalysis resulting in a decrease in ATP production and retarded cell growth.

The β -subunit of F_1 -ATPase from a wide variety of sources has been shown to be highly conserved at the amino acid sequence and comparison of the *S cerevisiae* β -subunit amino acid sequence with that from *E. Coli* confirms that the lysine and methionine residues shown by Parsonage et al to be very important for catalytic activity are conserved, with the lysine and methionine residues occurring at positions 196 and 255 respectively on the yeast β -subunit sequence.

In order to carry out SDM the wild-type β -subunit gene from yeast was isolated from the plasmid pGR208 (Figure 34) as an EcoRI/BamHI fragment which was cloned into M13mp19. Two
5 mutated β -subunit genes were constructed: mutant BB1 has both the Met255 and Lys196 converted to isoleucine and glutamine respectively while mutant BB2 has only the lysine to glutamine mutation (Figure 34). Following sequence analysis to ensure
10 correct mutagenesis with no unwanted mutations, the mutated β -subunit genes were removed from mp19 by EcoRI/BamHI digests. The fragments containing the genes were then blunt-ended and ligated to BglII digested pKV49 (Figure 35) which had previously
15 been blunt-ended. We have both mutated β -subunit genes cloned into pKV49 (pKV49-BB1 and pKV49-BB2) and have transformed the yeast strain BET9 with both these constructs. Growth curve and plate growth from both mutated β -subunit transformants
20 show tht the transformants have altered growth characteristics which are consistent with a mitochondrial defect.

Concurrently with the transformation of strain BET9 with the mutated β -subunit genes, gene
25 disruption may be used to construct a derivative of strain BET9 which will fail to synthesize β -subunit. The resultant strain will therefore be unable to grow on non-fermentable carbon sources although it will be easily maintainable on a
30 fermentable carbon source such as glucose. Transformation of this strain with plasmids bearing the mutated β -subunit genes, followed by measuring the transformants' growth characteristics on a non-fermentable carbon source, shows that the

altered β -subunit is unable to support oxidative phosphorylation.

EXAMPLE 4

FUSION PROTEINS

5 An alternative strategy for selectively
perturbing mitochondrial function is the expression
of a fusion protein which results in either poor or
no yeast cell growth. The candidate fusion protein
chosen from this project contains the N-terminal
10 region of the yeast ATP synthase β -subunit fused to
most of β -galactosidase from E. coli and has been
constructed by gene fusion (Figure 36). This
 β -subunit/ β -galactosidase fusion protein has
already been shown to be targeted to the inner
15 membrane of yeast mitochondria 921) and cells
expressing this fusion protein appear to be unable
to grow on a non-fermentable carbon source. In the
presence of the fusion protein the transducing
capacity of the mitochondrial membrane as measured
20 by the ^{32}P -ATP exchange reaction is only 9% of that
measured in the absence of the fusion protein. As
yet the mechanism of this description has not been
evaluated but the gene fusion is thought to produce
a protein which becomes trapped in the inner
25 membrane and interferes with function(s) essential
for respiratory growth.

CONSTRUCTION OF THE ATP2/LacZ GENE FUSION

30 The plasmid pGR208, which contains the yeast
ATP2 DNA encoding ATP synthase β -subunit gene
(Figure 34), was digested with EcoRI plus BamHI
resulting in the release of a 1.1kb fragment coding
for the first 350 amino acids of the β -subunit
protein. pMUR1720 is a pUC8 based plasmid which
contains a LacZ gene contained within an EcoRI/NarI

fragment (Figure 36). Cloning of the 1.1kb
EcoRI/BamHI DNA fragment coding for the first 350
amino acids of the yeast β -subunit protein into the
EcoRI/BamHI sites of pMUR1720 (Figure 36A) results
5 in an in-frame fusion between the 350 amino acids
of the β -subunit and the entire (minus the first
eight amino acids) LacZ protein (Figure 36). The
entire β -subunit/LacZ gene fusion is now contained
on the 4.3kb EcoRI/NarI fragment in construct
10 pMUR1720-BLZ (Figure 36). This 4.3kb EcoRI/NarI
fragment is currently being cloned into the pKV49
vector resulting in the pKV49-BLZ construct (Figure
37) which can be used to transform the yeast
strains BET9 and BE27 and show that when induced by
15 galactose growth defects consistent with
mitochondrial inhibition arise.

EXAMPLE 5

Construction of a promoter fusion between the MS10 gene and the UCP gene

20 The 1830 bp HindIII to BamHI fragment from
pMS10 was ligated into the binary plant
transformation vector Bin19 previously cut with
HindIII and BamHI.

Following ligation the resultant plasmid was
25 cut with BamHI and ligated to the 930 bp UCP BamHI
fragment from plasmid pUC/UCP (a derivative of
pUC19 containing the modified UCP gene cloned at
the BamHI site) to construct a fusion between the
MS10 gene promoter and the UCP gene. Finally the
30 nos 3' terminator obtained as a 250 bp SstI-EcoRI
fragment from vector pTAK1 was ligated into the
MS10-UCP construct previously cut with SstI and
EcoRI.

The resulting plasmid is termed pBin/MS10-UCP (Figure 39) and contains the MS10 promoter, the UCP gene, nos 3' terminator expression cassette located between the right and left border sequences of Agrobacterium T-DNA allowing efficient transformation into tobacco cells.

EXAMPLE 6

Transformation of tobacco plants with pBin/MS10 promoter gene constructs

The recombinant vector pBin/MS10-UCP was mobilised from E Coli (TG-2) onto Agrobacterium tumefaciens (LBA4404) in a triparental mating on L-plates with E Coli (HB101) harbouring pRK2013. Transconjugants were selected on minimal medium containing kanamycin ($50\mu\text{g}/\text{cm}^3$) and streptomycin ($500\mu\text{g}/\text{cm}^3$).

L-Broth (5 cm^3) containing kanamycin at $50\text{ g}/\text{cm}^3$ was inoculated with a single Agrobacterium colony. The culture was grown overnight at 30°C with shaking at 150 rpm. This culture ($500\mu\text{l}$) was inoculated into L-Broth containing kanamycin ($50\mu\text{g}/\text{cm}^3$) and grown as before. Immediately before use the Agrobacteria were pelleted by spinning at 3000 rpm for 5 minutes and suspended in an equal volume of liquid Murashige and Skoog (MS) medium.

Feeder plates were prepared in 9 cm diameter petri dishes as follows. Solid MS medium supplemented with 6-benzyl-aminopurine (6-BAP) (1 mg/1) and 1-naphthaleneacetic acid (NAA) (0.1 mg/1) was overlaid with Nicotiana tabacum var Samsun suspension culture (1 cm^3). One 9 cm and one 7cm filter paper discs were placed on the surface.

Whole leaves from tissue culture grown plants were placed in the feeder plates. The plates were

sealed with "Nescofilm" (Trade Mark) and incubated overnight in a plant growth room (26°C under bright fluorescent light).

5 Leaves from the feeder plates were placed in
 Agrobacteria suspension in 12 cm diameter petri
 dishes and cut into 1- 1.5 cm² sections. After 20
 minutes the leaf pieces were returned to the feeder
 plates which were sealed and replaced in the growth
10 the plant material was transferred to MS medium
 supplemented with 6-BAP (1 mg/l), NAA (0.1 mg/l),
 carbenicillin (500µg/cm³) and kanamycin (100
 µg/cm³), in petri dishes. the petri dishes were
 sealed and returned to the growth room.

15 Beginning three weeks after inoculation with
 Agrobacterium, shoots were removed from the
 explants and placed on MS medium supplemented with
 carbenicillin (200 µg/cm³) and kanamycin
 (100µg/cm³) for rooting. Transformed plants rooted
20 1-2 weeks after transfer.

 Following rooting, transformed plants were
 transferred to pots containing soil and grown in
 the glasshouse. Roughly one month after transfer
 the plants flowered.

25 The anthers of the tobacco plants containing
 the pBin/MS10-UCP construct were were assayed for
 expression of the UCP gene by Northern blotting of
 RNA samples, and the effect of UCP expression on
 pollen development determined.

30

1. A plant gene construct comprising a
disrupter gene encoding a protein which is
capable of disrupting the biogenesis of viable
pollen, and a gene regulatory sequence which
5 includes a promoter sequence inducible by
external application of an exogenous chemical
inducer to a plant containing the construct.

2. A plant gene construct as claimed in claim
1 in which the regulatory sequence includes an
operator sequence controlling said disrupter
gene and a repressor gene encoding a repressor
5 protein adapted to interact with the disrupter
gene operator and inhibit expression of the
disrupter protein, said repressor protein gene
being regulated by the chemically inducible
promoter.

3. A plant gene construct as claimed in claim
1 or claim 2 in which the construct also
includes a male flower specific promoter
sequence operatively linked to said disrupter
5 protein gene, for restricting expression of
the disrupter protein gene to male flower
parts of a plant.

4. A plant gene construct as claimed in claim 1 or 2 or 3 in which the construct comprises:

5 (a) a first gene promoter sequence responsive to the presence or absence of an exogenous chemical inducer,

(b) a gene encoding a repressor protein under control of the said first promoter sequence;

10 (c) an operator sequence responsive to the said repressor protein expressed by the repressor protein gene;

(d) a male flower specific gene promoter sequence expressible only in male parts of a plant; and,

15 (e) a gene encoding a disrupter protein capable of disrupting biogenesis of viable pollen;

20 whereby the presence or absence of the exogenous chemical inducer in the plant enables selection of male fertility or sterility.

5. A plant gene construct as claimed in any of claims 1 to 4 in which the chemically inducible gene promoter sequence is a 27kd subunit isolated from the maize glutathione-S-transferase (GST II) gene.
- 5

6. A plant gene construct as claimed in claim 5, in which the inducer for the chemically inducible gene promoter is N,N-diallyl-2,2-dichloro-acetamide or benzyl-2-chloro-4-(tri-fluoro- methyl)-5-thiazole-carboxylate.
7. A plant gene construct as claimed in claim 6 in which the inducer is N,N-diallyl-2,2-dichloro-acetamide.
8. A plant gene construct as claimed in any of claims 1 to 7 in which the disrupter gene is the mammalian uncoupling protein (UCP) gene.
9. A gene construct as claimed in any of claims 1 to 7, in which the disrupter gene is a mutated form of the gene for the β -subunit of F_1 -ATPase which has sequences added or deleted such that these changes result in the retention of the ability to assemble with other subunits but interfere with function as an ATP synthase.
10. A plant gene construct as claimed in any of claims 1 to 7, in which the disrupter gene is a mutated, synthetic form of the oli 1 gene encoding subunit 9 of the F_o -ATPase.

11. A plant gene construct as claimed in any of claims 1 to 7, in which the disrupter gene is a mutated form of a mitochondrial transit pre-sequence which malfunctions during transfer resulting in the disruption of protein transport to mitochondria.
12. A plant gene construct as claimed in any of claims 1 to 7 in which, the disrupter gene is a gene construct carrying a fusion between the β -subunit gene from yeast and the β -galactosidase gene from E. coli, resulting in expression of a disrupting fusion protein.
13. A plant gene construct as claimed in any of claims 2 to 12, in which the repressor gene is a DNA-binding protein gene placed under control of the chemically inducible promoter, the encoded DNA-binding protein gene being capable of interaction with the operator sequence associated with the disrupter gene.
14. A plant gene construct as claimed in claim 13, in which the DNA-binding protein gene encodes a repressor protein so that on expression of the DNA-binding protein gene expression of protein by the disrupter gene is inhibited.

15. A plant gene construct as claimed in claim 12 or 13, in which both the DNA-binding protein gene and the said operator are of non-plant origin.
16. A plant gene construct as claimed in claim 15, in which both the DNA-binding protein gene and the said operator are of bacterial origin.
17. A plant gene construct as claimed in claim 16, in which the repressor gene and the operator are derived from the lacI gene of Escherichia coli.
18. A plant gene construct as claimed in claim 17, in which the repressor and operator are isolated from the plasmid, designated p35SlacI, deposited in an Escherichia coli, strain TG-2, host with the National Collection of Industrial and Marine Bacteria Limited, Aberdeen, United Kingdom, on 12th December 1988, under the Accession Number NCIB 40092.
19. A plant gene construct as claimed in any of claims 2 to 12 in which the disrupter gene operator is a pseudo-operator sequence, and the repressor gene is a mutant regulatory gene encoding a repressor having an amino acid sequence which binds to the pseudo-operator.

20. A plant gene construct as claimed in any of claims 3 to 19 in which the male flower specific sequence is the polynucleotide shown in Figure 15 herewith, which is specifically expressed in male flower tissue or a variant thereof permitted by the degeneracy of the genetic code.
21. A plant gene construct as claimed in claim 20, in which the male flower specific sequence is isolated from plasmid pMS10 which has been deposited in an Escherichia coli strain R1 host with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40090.
22. A plant gene construct as claimed in claim 21 or 22 in which the male flower specific and disrupter gene are isolated from the plasmid designated pBin/MS10-UCP having the structure shown in Figure 39 of the accompanying drawings.
23. A plant gene construct as claimed in any of claims 3 to 19, in which the male flower specific sequence comprises the polynucleotide shown in Figure 16 herewith, which is specifically expressed in male flower tissue or a variant thereof as permitted by the degeneracy of the genetic code.

24. A plant gene construct as claimed in claim 23, in which the male flower specific gene is isolated from the plasmid pMS14 which has been deposited in an Escherichia coli strain DH5 α host, with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40099.
25. A plant gene construct as claimed in any of claims 3 to 19, in which the male flower specific sequence comprises the polynucleotide shown in Figure 17 herewith, which is specifically expressed in male flower tissue or a variant thereof permitted by the degeneracy of the genetic code.
26. A plant gene construct as claimed in claim 25, in which the male flower specific sequence is isolated from the plasmid pMS18 which has been deposited in an Escherichia coli strain R1 host, with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40100.
27. A transformed plant and plant parts such as cells, protoplasts and seeds incorporating the construct claimed in any of the preceding claims.

28. A plant which is restorably male sterile,
in which said plant contains, stably
incorporated in its genome by transformation,
the recombinant plant gene construct claimed
in any of the preceding claims.

5

29. A plant or plant part as claimed in claim
27 or 28, in which the plant is
dicotyledonous.

30. A plant or plant part as claimed in claim
27 or 28, in which the plant is
monocotyledonous.

1/38

RESTORABLE MALE STERILITY

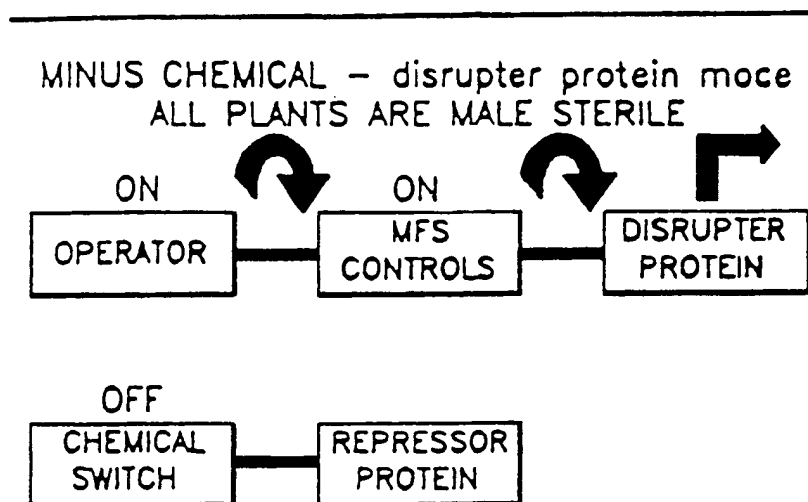


FIG. 1.

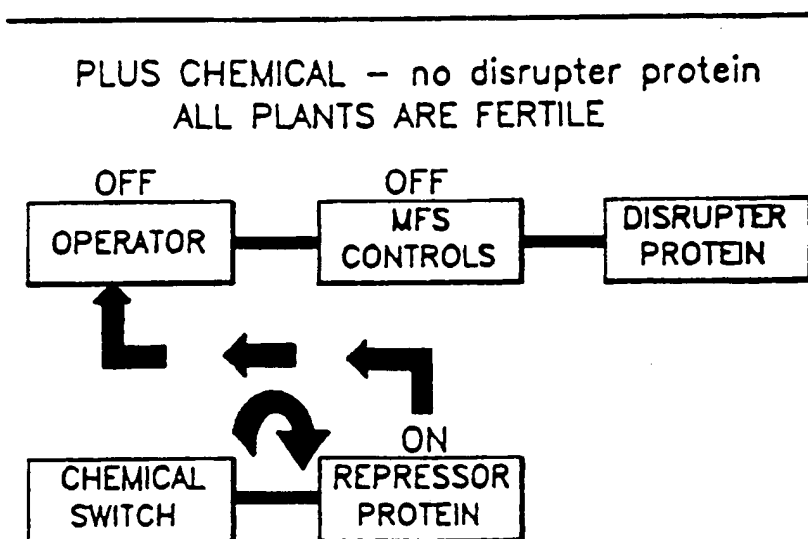
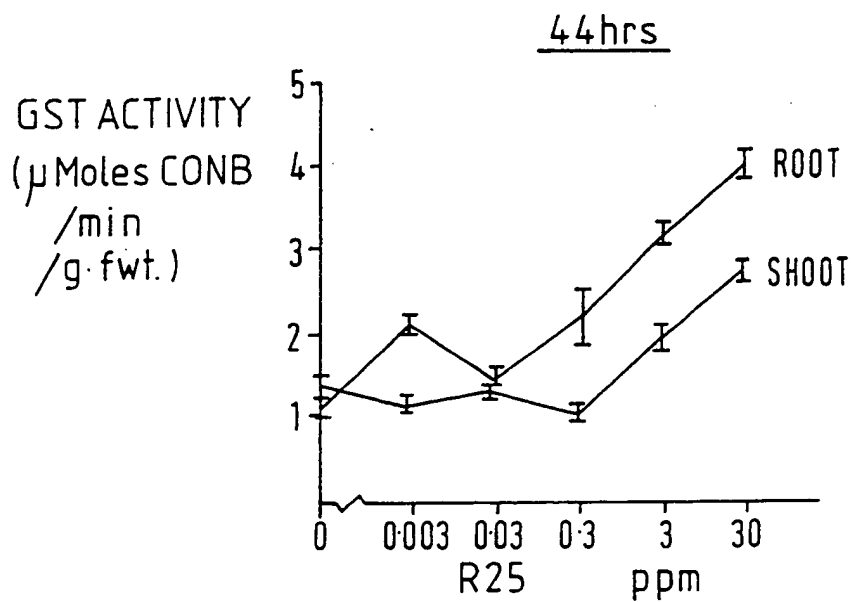
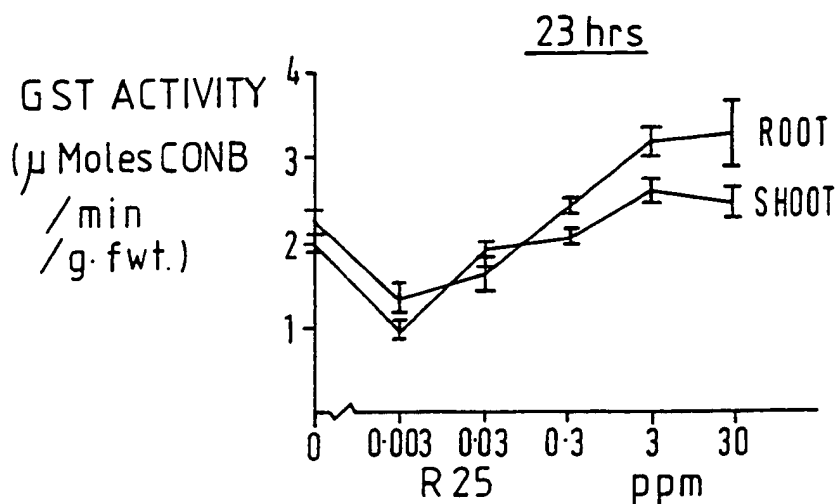


FIG. 2.

SUBSTITUTE SHEET

2/38

FIG. 3.

Stimulation of GST activity in roots and shoots by safener treatment

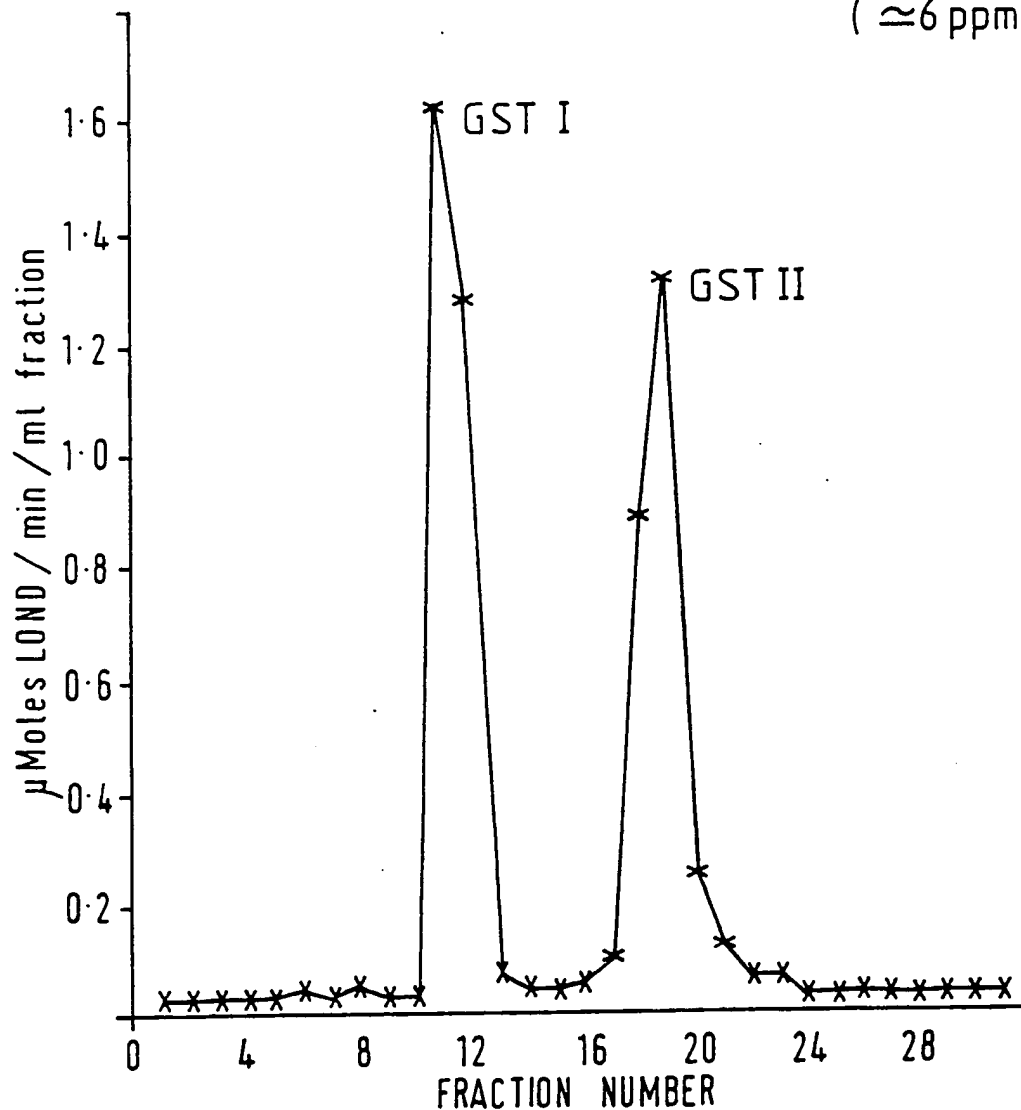
SUBSTITUTE SHEET

3/38

FIG. 4.

Separation of GST I and GST II in roots tissues treated with R25

GST FROM MAIZE ROOTS TREATED WITH R25

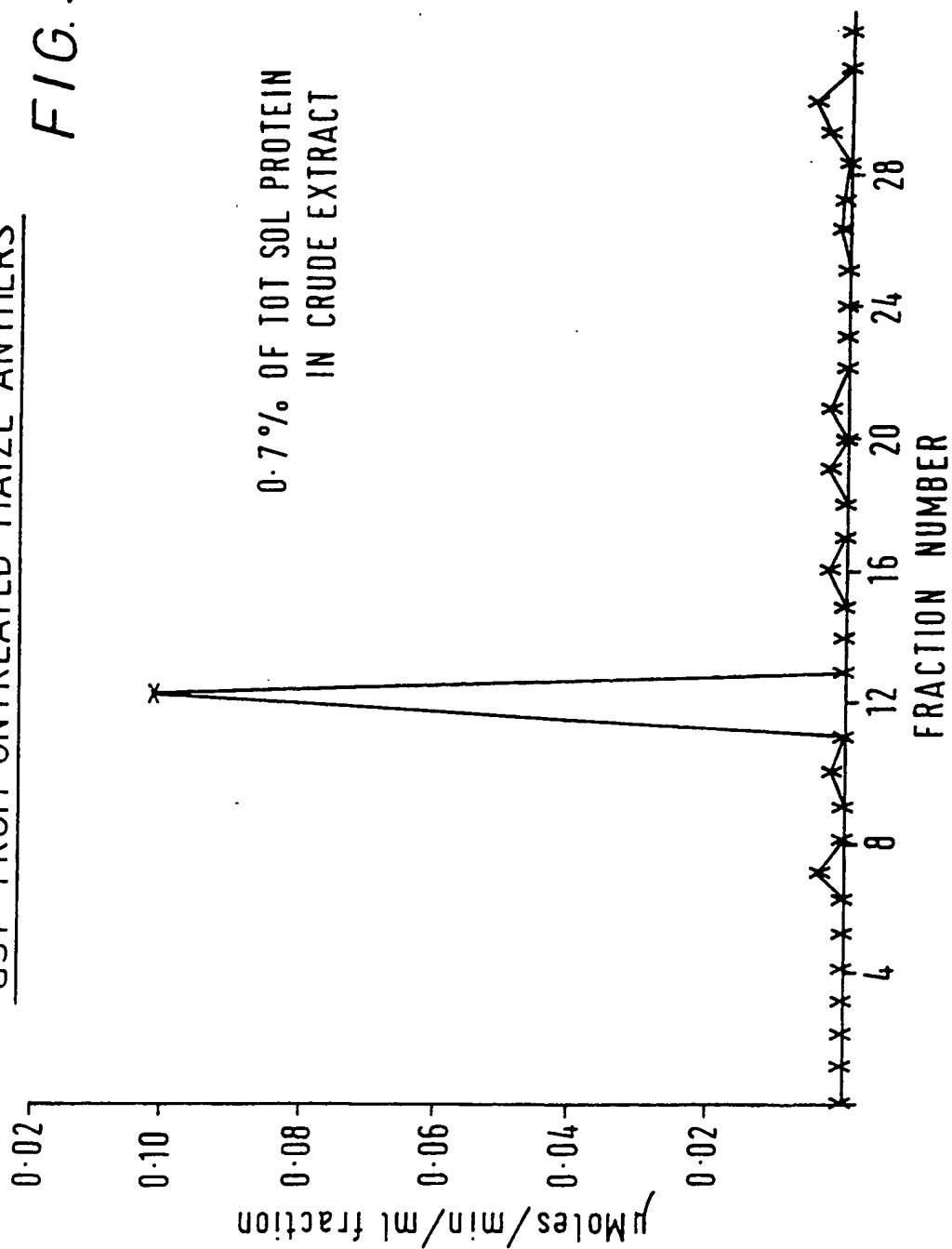
(≈ 6 ppm)GST I $\approx 0.9\%$ OF TOTAL SOL PROTFINGST II $\approx 2.5\%$ OF TOTAL SOL PROTFIN

[USING DATA FROM MOZER (MONSANTO)]

SUBSTITUTE SHEET

4/38

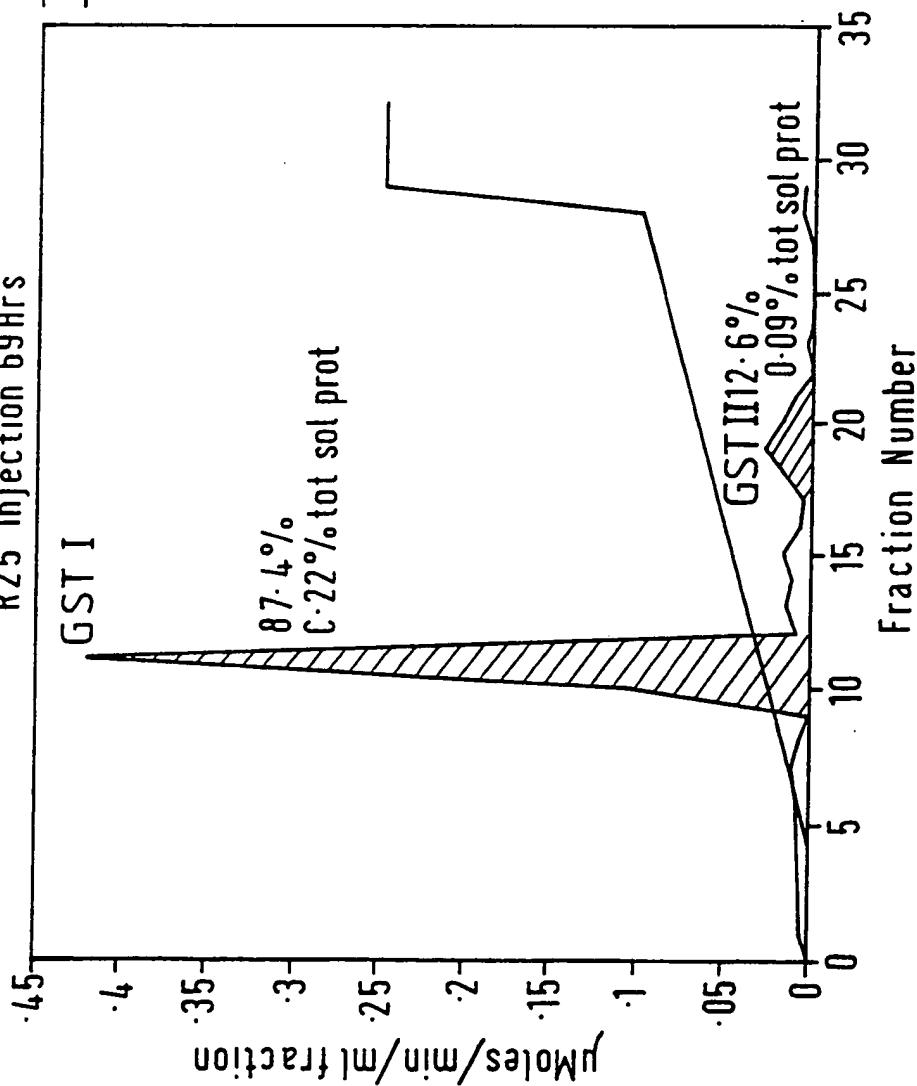
GST I is the only isoform present in untreated anthers
GST FROM UNTREATED MAIZE ANTHERS
 FIG. 5.



5/38

GST II activity is stimulated in anthers treated with R 25

GST ISOFORM SEPARATION R25 Injection 69Hrs



SUBSTITUTE SHEET

6/38

GST II activity is stimulated in anthers fed with R25 from a stem reservoir

GST ISOFORM SEPARATION R25 Reservoir (injection) 60Hrs

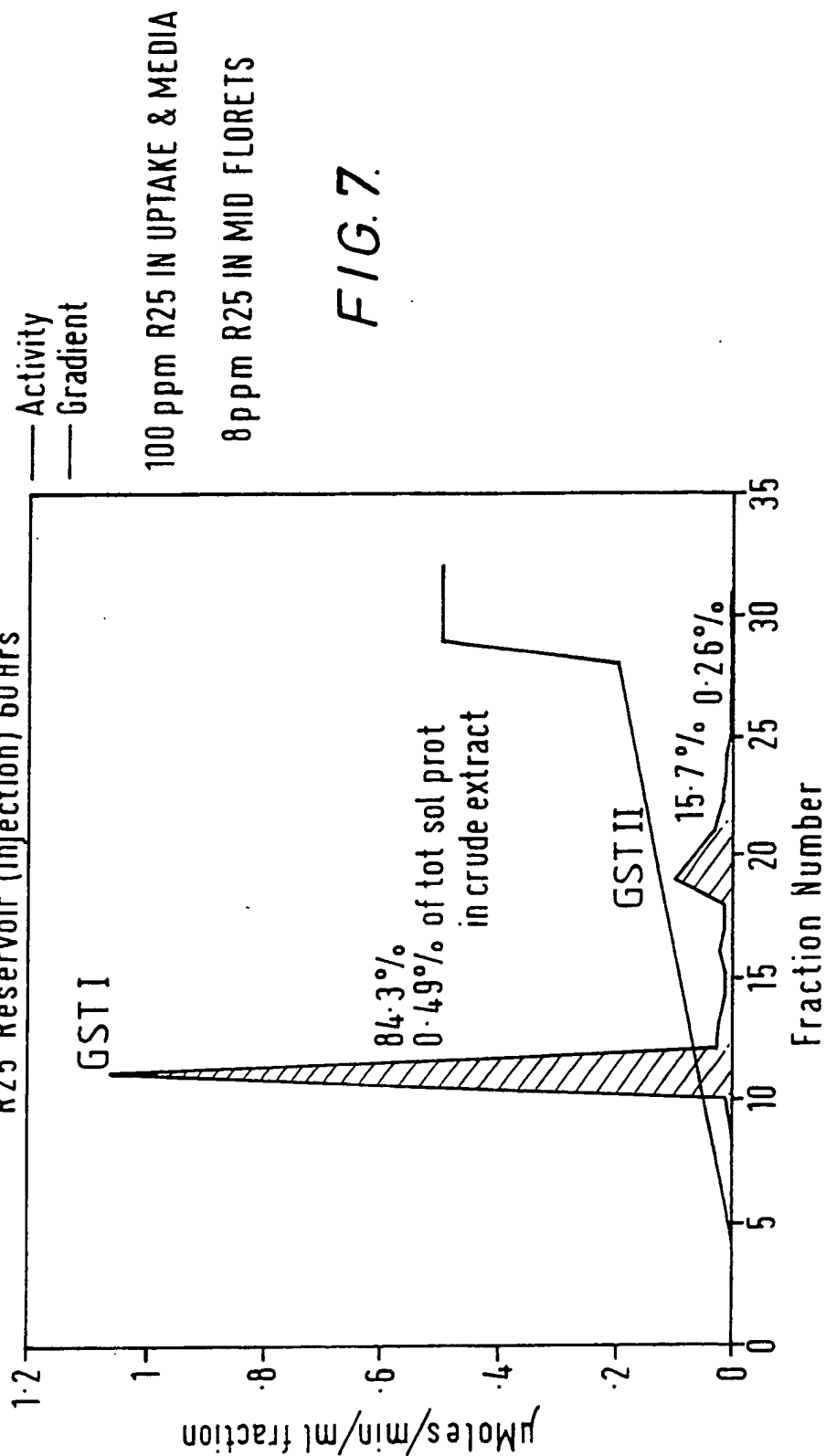
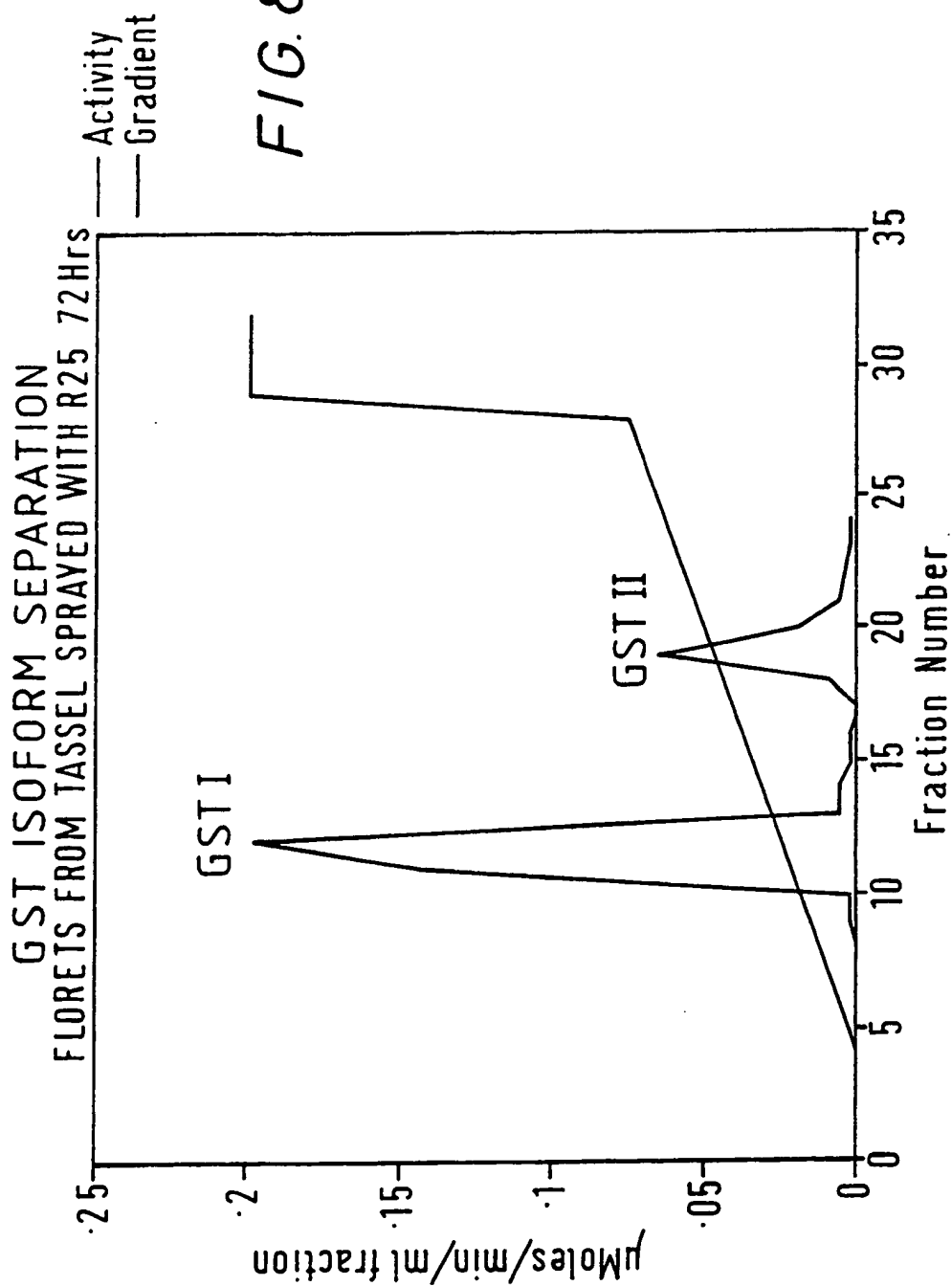


FIG. 7.

7/38

GST II activity is stimulated in anthers sprayed directly with R25

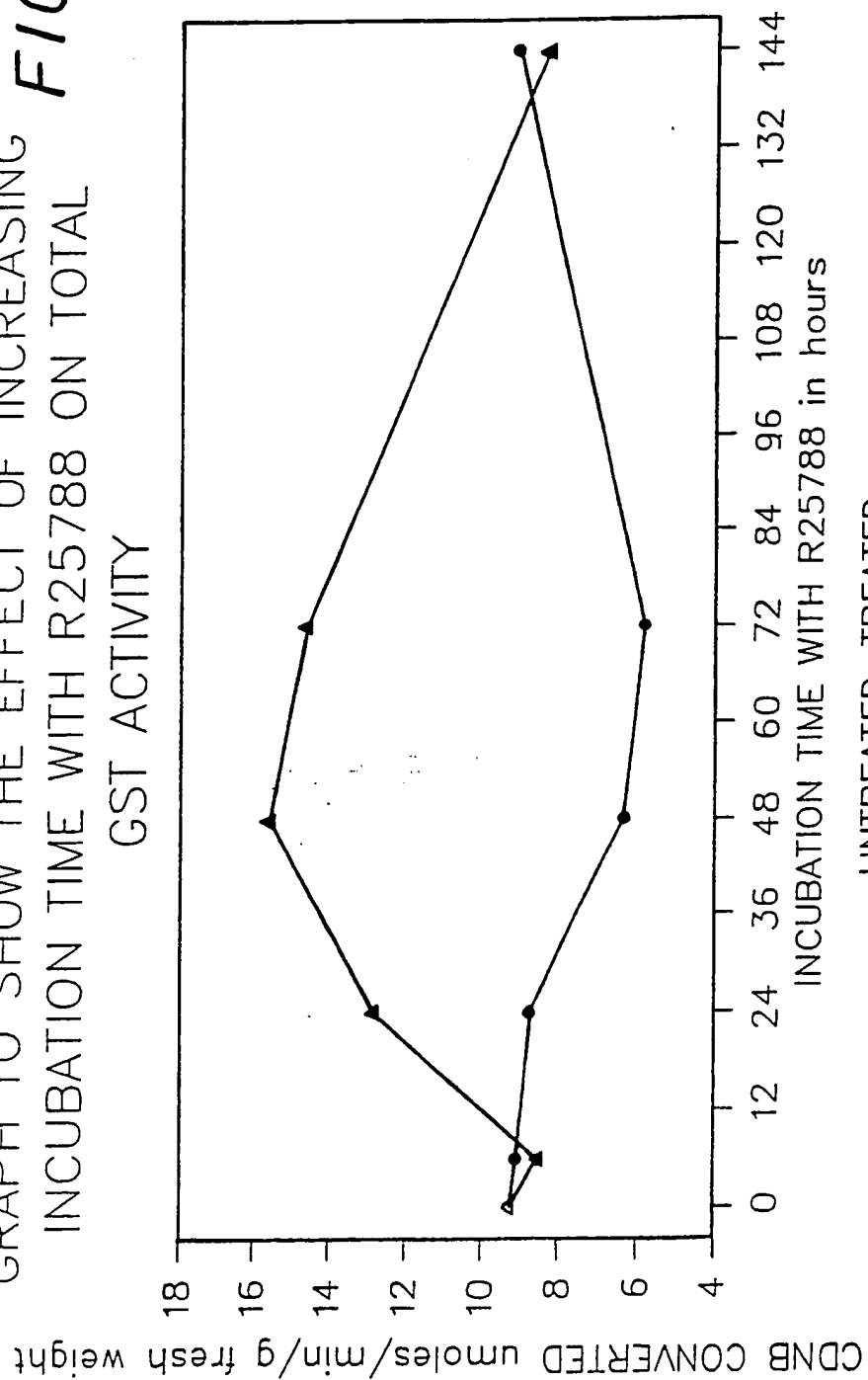


SUBSTITUTE SHEET

8/38

GRAPH TO SHOW THE EFFECT OF INCREASING
INCUBATION TIME WITH R25788 ON TOTAL
GST ACTIVITY

FIG. 9.

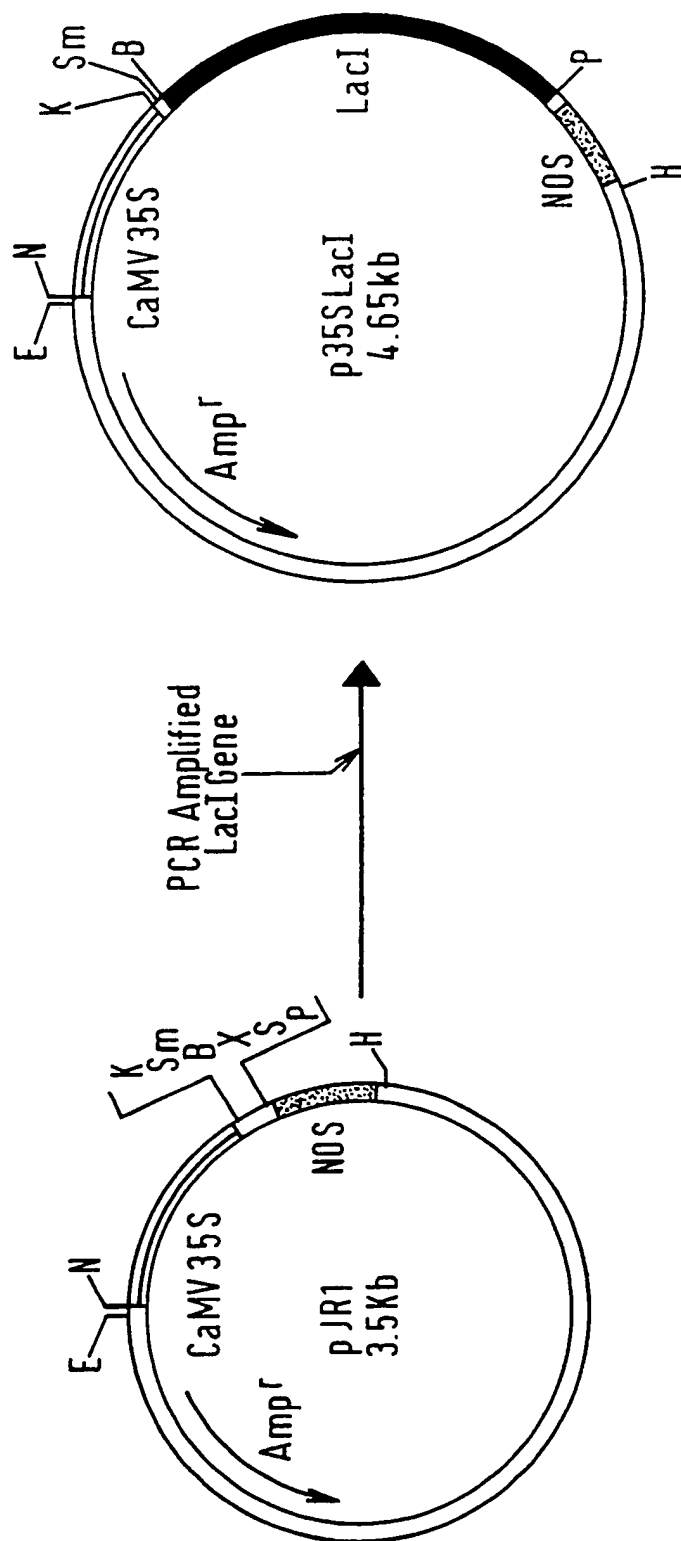


UNTREATED TREATED

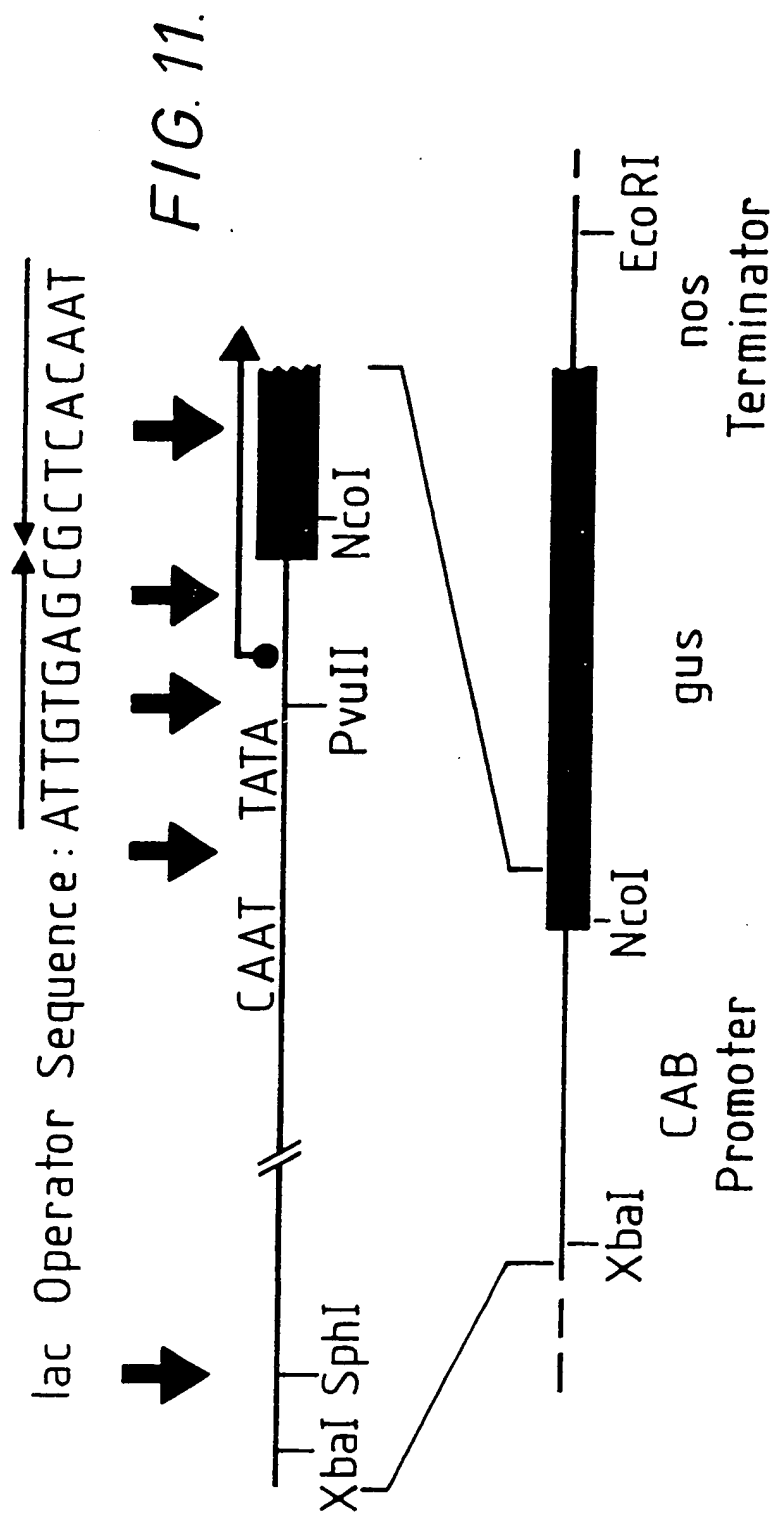
Approx 2.5 fold increase in total GST activity
at 48 hours.

SUBSTITUTE SHEET

9/38

FIG. 10. Cloning PCR-amplified lacI**SUBSTITUTE SHEET**

10/38



SUBSTITUTE SHEET

11/38

SEQUENCE OF THE XbaI - NcoI CAB PROMOTER FRAGMENT.

	10	20	30	40	50
XbaI					
1	GTCTAGATATGTTTCTCTTTTGGTTCTTGAGTTGACAAGTGGCATGCTAT				
51	TTTTGCTCATGTGAGAAAAAATATAGCACCGTTTGTTCGTTGAAATTGA				
101	ATTGCATCCCAATAATCATAATTTAGACATAAACCAACTAAGTTAATATA				
151	TTTGTATATGTAAATATGTTATCCTAAATTATAATTATATGAGAGAGATA				
201	GTTATACATTACAATTATGATATAGAGAAGCAAATAGAAGAGTGTGCTAT				
251	AAGTTGTACATTGTAAAAGTAGTATGCAAATTTATAGAATTAATTTTAT				
301	CTTTCACCTCATAAATTTAAGATACACTTATATATAAACTTTGAAAAGTT				
351	GTA AA AATATCATATTCTAAAA AA AATAGACTATTCTATTAGTTAGATTCC				
401	AATTCCTTAAAATAAAAGAAAATAAATGGGCTTAAGGGCTAGTTTGGTGG				
451	GCAGGTGGAGGCGATCAATGGCGGATGAATCCTCTCGATATTCAATTGAG				
501	GGGGTTCATCCCATGAATCTCTCTATTTACATGTCCACCAAACAAGATTA				
551	AATGCAATGATACATAGCTTTTGTGCATATTTTTTTTTTAACGGAGATGCA				
601	AAACAGTATACAAGATGTGTGTATGTTCCCATGCGACCCATCTTGTATCC				
651	AGTTCATGCGTCTGGTGGTTGAACCAGAAAGATACACATGATCCATTTCCG				
701	ATGAATCAACACAAAAGACTTCATTGATTATGCATTTGACAAATAGCAAG				
751	AAGCGCGGGGCAACAAAGCCCATCCTATCTCATCCATTTTTTCCCACGAT				
801	GGCAAGTGGCAGCTCCTGATTAGCTACGCCCATTCCTATGCTATGTGGCA				
851	CACCCCAGGATTCTTGTGTGATAGGCCATTGGGGCCACGAGGAGCCACGT				
901	CAGACGCCAAGCCACCCCGGCGAGACCAACCAGCCAATCGCAGTTCAGGA				
951	AAAGATGACCTGCTATCCAAACCCAACTGTATAAAAGGCAGCTGCTGTGT				
	* * * *				
1001	TCTGTTATGACACAGCCATCACACGCATACTGCATACACAACACAGAGCA				
		M A A A T M			
1051	TCAGAAGGAGCTACGATCTGATCGACATGGCTGCCGCCACCATGG				
		NcoI			

_____ = CAT and TATA sequences.

* = Possible transcription start sites.

FIG. 12.

SUBSTITUTE SHEET

12/38

434 REPRESSOR GENE		SEQUENCE AT <i>Sal</i> I SITE	
PRESENT	ABSENT		
pPS1	pAD18	GTCGAACAAGAAAGTTTGTTCGAC	WILD-TYPE 434 O _R 1
pPS2	pAD16	GTCGAACAATATATATTGTTCGAC	"WILD-TYPE" SYNTHETIC
pPS3	pAD17	GTCGATCAATATATATTGATCGAC	"MUTANT" SYNTHETIC
pAD15.2	pAD14	G.....TCGAC	NO OPERATOR

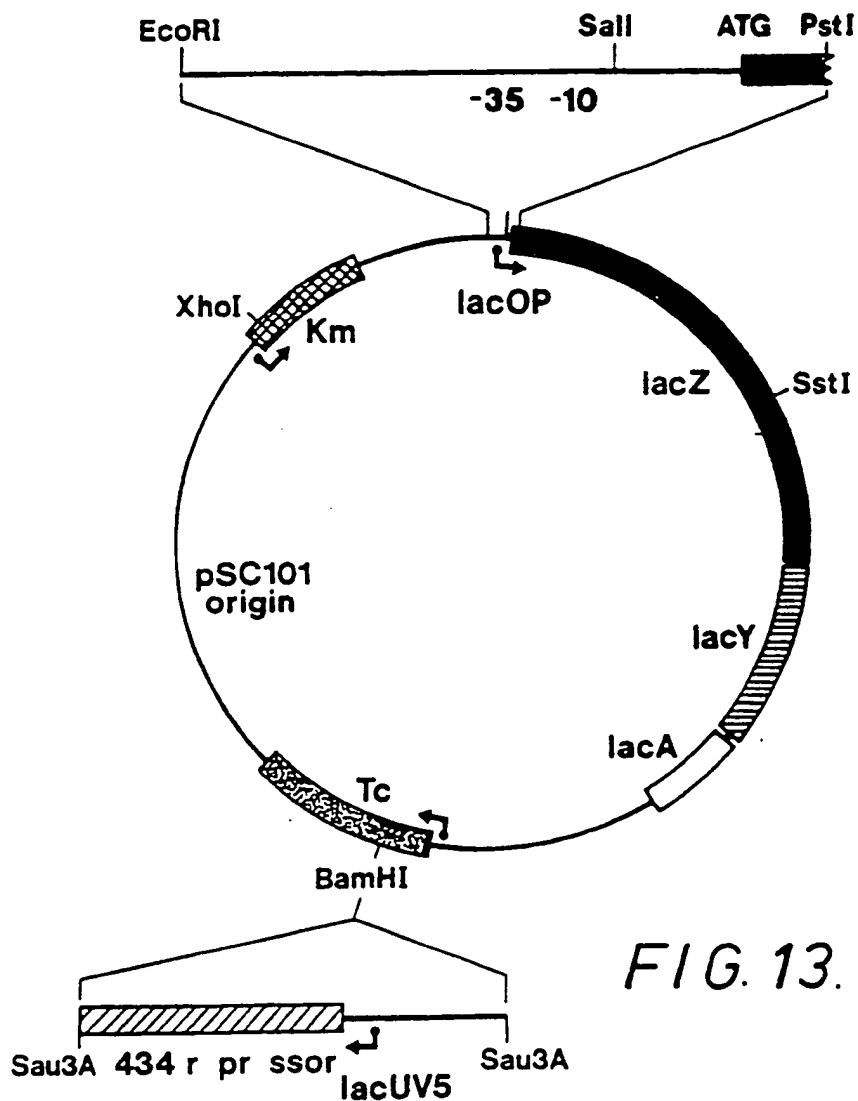
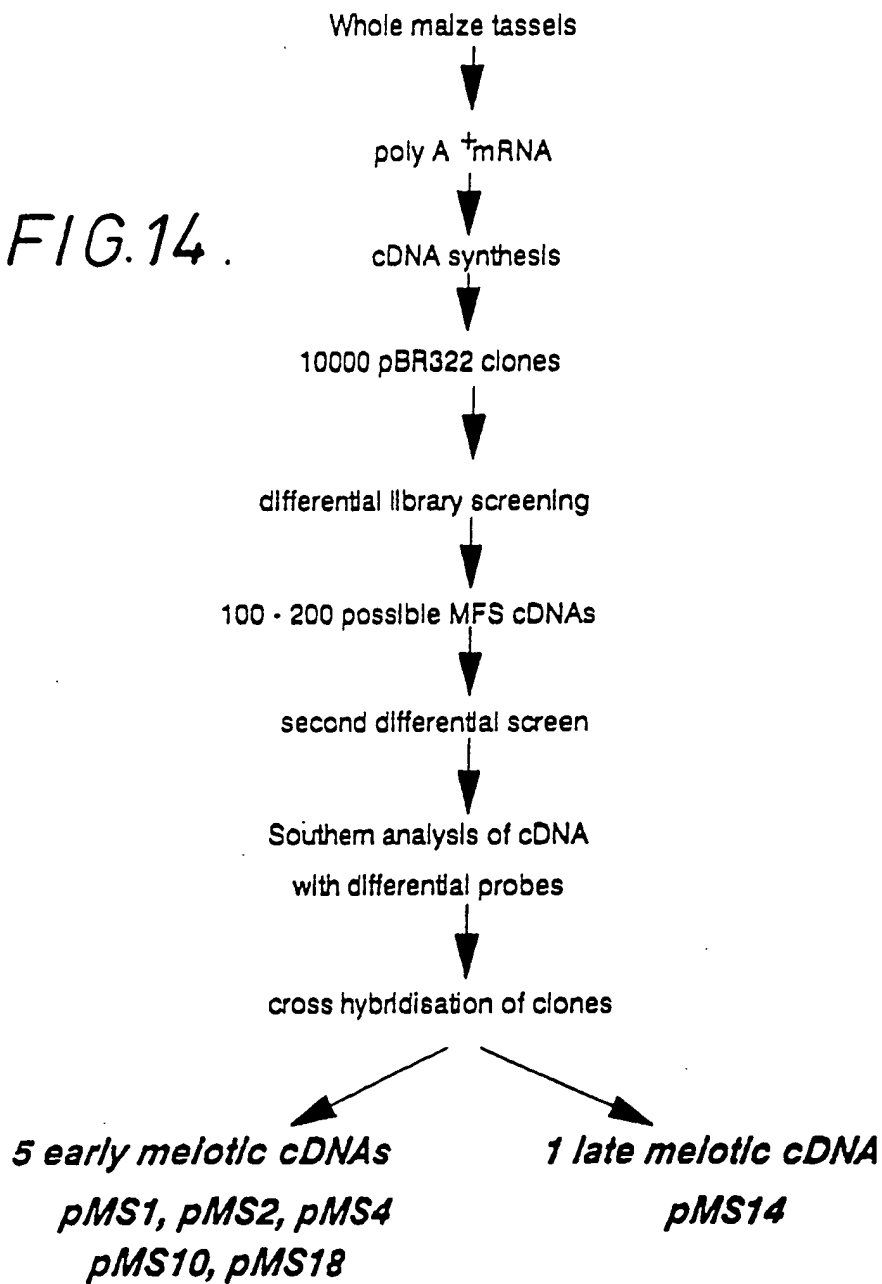


FIG. 13.

SUBSTITUTE SHEET

13/38

ISOLATION OF cDNA CLONES**FIG.14.****SUBSTITUTE SHEET**

14/38

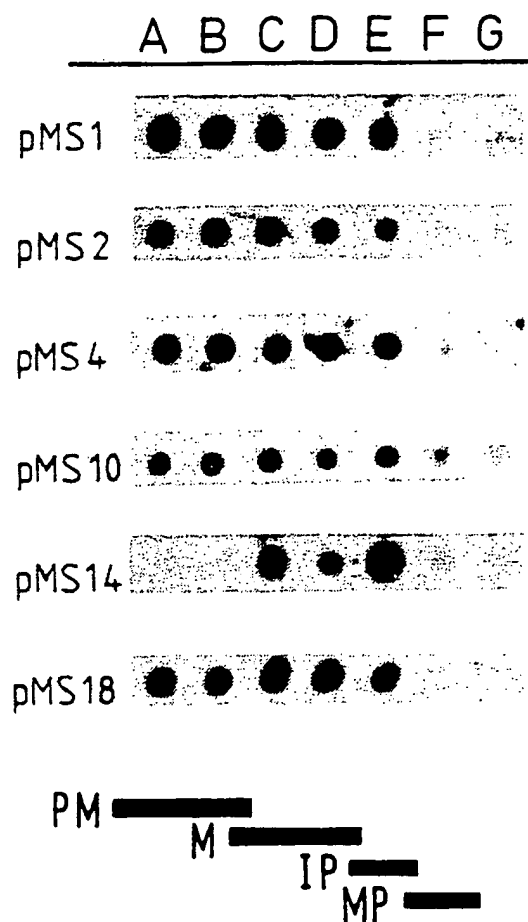


FIG. 15.

15/38

A



FIG. 16A.

SUBSTITUTE SHEET

16/38

B

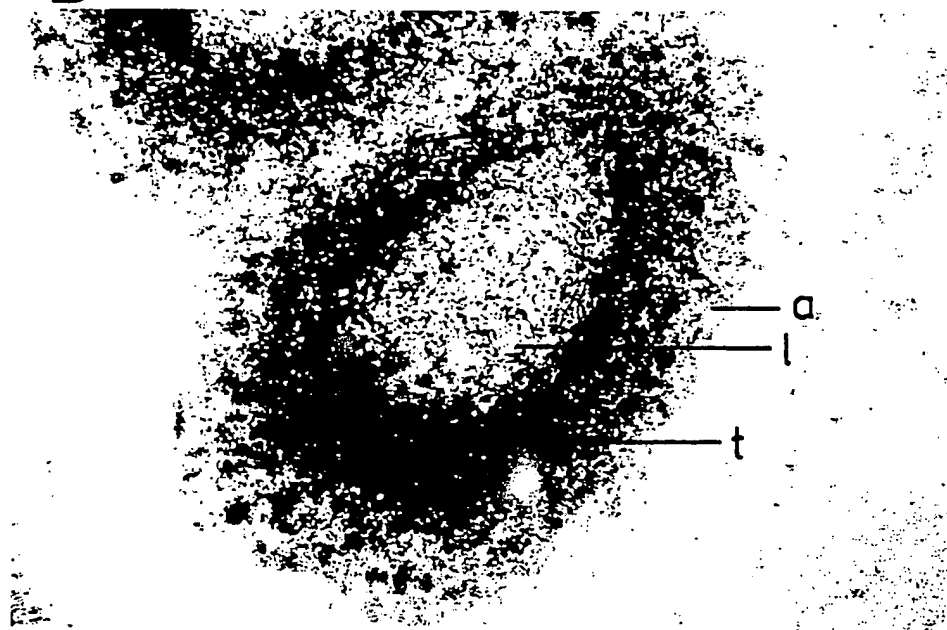


FIG. 16B.

C



FIG. 16C.

SUBSTITUTE SHEET

17/38

Nucleotide and deduced amino acid sequence for male flower specific cDNA clone, pMS10.

SUBSTITUTE SHEET

18/38

FIG. 17.
(cont.)

500	510	520	530	540
GTT CAT TAT AGC AGA CCA TCA AGA ACT GAC AGG AAC CGT AAC TAC				
Val His Tyr Ser Arg Pro Ser Arg Thr Asp Arg Asn Arg Asn Tyr				
550	560	570	580	
CGA GGA AAC TAC CAG GAT GGC CCT CCA CAG CAA GGA AAT TAC CAG				
Arg Gly Asn Tyr Gln Asp Gly Pro Pro Gln Gln Gly Asn Tyr Gln				
590	600	610	620	630
AAC AAC CGT CCT CCA CCA GAA GGT GGT TAC CAG AAC AAC CCG CCG				
Asn Asn Arg Pro Pro Pro Glu Gly Gly Tyr Gln Asn Asn Pro Pro				
640	650	660	670	
CAG CAA GGA AAC TAC CAG ACA TAC CGC TCG CAG CAA GAT GGA AGA				
Gln Gln Gly Asn Tyr Gln Thr Tyr Arg Ser Gln Gln Asp Gly Arg				
680	690	700	710	720
GGC TAT GCC CCA CAG CAG AAT TAT GCA CAA GGT GGT CAG GAT GGT				
Gly Tyr Ala Pro Gln Gln Asn Tyr Ala Gln Gly Gly Gln Asp Gly				
730	740	750	760	
AGA GGT TTT GGA AGG AAT GAT TAC ACA GAC CGT TCA GGC TAC AAT				
Arg Gly Phe Gly Arg Asn Asp Tyr Thr Asp Arg Ser Gly Tyr Asn				
770	780	790	800	810
GGA CCC ACT GAT TTT CGA AGT CAA ACT CAG TAC CAA GGG CAT GTA				
Gly Pro Thr Asp Phe Arg Ser Gln Thr Gln Tyr Gln Gly His Val				
820	830	840	850	
AAT CCA GCT GGG CAA GGT CAA GGT TAC AAC AAC CCC CAA GAG CGT				
Asn Pro Ala Gly Gln Gly Gln Gly Tyr Asn Asn Pro Gln Glu Arg				
860	870	880	890	900
ACG AAC TTC TCG CAA GGG CAG GGA GGA GGT TTT AGG CCT GGT GGT				
Thr Asn Phe Ser Gln Gly Gln Gly Gly Phe Arg Pro Gly Gly				
910	920	930	940	
CCT TCA GCA CCT GGG TCT TAT GGC CAA CCA TCA GCA CCT GGA TCT				
Pro Ser Ala Pro Gly Ser Tyr Gly Gln Pro Ser Ala Pro Gly Ser				
950	960	970	980	990
TAT GGT CAA CCT AAT ACA CTT GGT AAC TAT GGG CAG GTA CCT CCA				
Tyr Gly Gln Pro Asn Thr Leu Gly Asn Tyr Gly Gln Val Pro Pro				

SUBSTITUTE SHEET

19/38

FIG. 17.

(cont.)

1000	1010	1020	1030
TCA GTG AAT CCT GGT GGT AAC AGA GTT CCT GGT GTG AAT CCT AGT			
Ser Val Asn Pro Gly Gly Asn Arg Val Pro Gly Val Asn Pro Ser			
1040	1050	1060	1070
TAT GGT GGG GAT GGC AGA CAG GGG GCT GGA CCA GCA TAT GGT GGA			
Tyr Gly Gly Asp Gly Arg Gln Gly Ala Gly Pro Ala Tyr Gly Gly			
1090	1100	1110	1120
GAT AAC TGG CAA AGA GGT TCT GGT CAG TAT CCT AGC CCA GGT GAA			
Asp Asn Trp Gln Arg Gly Ser Gly Gln Tyr Pro Ser Pro Gly Glu			
1130	1140	1150	1160
GGA CAA GGA AAC TGG CAG GGA AGG CAG TAA GAG CTG ACG TGT TCC			
Gly Gln Gly Asn Trp Gln Gly Arg Gln			
1180	1190	1200	1210
ACT GAA GAC AAG AAT GGC ACT TGA GAT TTA GAA ATC TCC ATC TGT			
1220	1230	1240	1250
AAA ATA AAC GAC TGT GAT GCA TTA CTC TTT TTT TTT TTC TTG CAT			
1270	1280	1290	1300
TTG AAC TCT AAA CTT ATG GGC ATG CGT TAT TAC CAA ACT ACG GAT			
1310	1320	1330	1340
GCA AAT TCA TTT TAG TTT TTT GGG CCA AAT GTT GGC ATT TTT AAA			

AAA

SUBSTITUTE SHEET

20/38

FIG. 18.

Nucleotide and deduced amino acid sequence for the male flower specific cDNA clone, pMS14.

	10		20		30		40							
GCA	GGG	GGG	GGG	GCA	CAG	CAA	GCC	AGC	AGA	GCA	GAA	AGC	AGC	CGC
Ala	Gly	Gly	Gly	Ala	Gln	Gln	Ala	Ser	Arg	Ala	Glu	Ser	Ser	Arg
	50		60		70		80		90					
AGC	CCC	AGC	CCC	CAC	AAA	GAC	GAA	GGC	AAC	AAT	GGC	GCT	AGA	AGC
Ser	Pro	Ser	Pro	His	Lys	Asp	Glu	Gly	Asn	Asn	Gly	Ala	Arg	Ser
	100		110		120		130							
AGC	CAC	GCC	CCC	CGC	GCA	CTC	CTC	GCG	CGT	GCC	TCG	TCC	TGC	TGG
Ser	His	Ala	Pro	Arg	Ala	Leu	Leu	Ala	Arg	Ala	Ser	Ser	Cys	Trp
	140		150		160		170		180					
TCC	TCG	GCG	GCG	GCA	CCG	GCC	CGT	CGT	CGG	TGC	TCA	GCG	CGC	CGG
Ser	Ser	Ala	Ala	Ala	Pro	Ala	Arg	Arg	Arg	Cys	Ser	Ala	Arg	Arg
	190		200		210		220							
GGC	GCA	GGA	CCG	GCG	GCA	GTG	CCT	GCC	GCA	GCT	GAA	CGC	CTC	CTG
Gly	Ala	Gly	Pro	Ala	Ala	Val	Pro	Ala	Ala	Ala	Glu	Arg	Leu	Leu
	230		240		250		260		270					
CGG	TGC	CGC	GCG	TAC	CTG	GTG	CCG	GCG	CGC	CGG	ACC	CCA	GCG	CGG
Arg	Cys	Arg	Ala	Tyr	Leu	Val	Pro	Ala	Arg	Arg	Thr	Pro	Ala	Arg
	280		290		300		310							
ACT	GCT	GCA	GCG	CTG	ACG	CGC	CGT	GTG	CAC	GAG	TGC	GCC	TGC	AGC
Thr	Ala	Ala	Ala	Leu	Thr	Arg	Arg	Val	His	Glu	Cys	Ala	Cys	Ser
	320		330		340		350		360					
ACC	ATG	GGC	ATC	ATC	AAC	AGC	CTG	CCC	GGC	CGG	TGC	CAC	CTC	GCC
Thr	Met	Gly	Ile	Ile	Asn	Ser	Leu	Pro	Gly	Arg	Cys	His	Leu	Ala
	370		380		390		400							
CAA	GCC	AAC	TGC	TCC	GCT	TGA	AGC	AGG	GAC	CTG	GCA	CGC	GTG	CTG
Gln	Ala	Asn	Cys	Ser	Ala									
	410		420		430		440		450					
CAA	TGG	ATG	GCA	GGA	GGG	GAG	AGG	AAT	AAG	AAG	TGT	TTC	CAT	TTC
	460		470		480		490							
ACA	GTG	AGA	GCA	GTC	GAG	CTC	CAA	CGT	TGT	CGT	CGT	CGT	CGT	CTT

SUBSTITUTE SHEET

2/38

FIG. 18.

(cont.)

500		510		520		530		540						
CTT	CTT	TTG	ATA	TTC	AGA	CTC	TGT	CTT	GCG	GTC	TAT	ATC	ATC	AGC
	550		560		570		580							
ATA	ATA	ATA	ATA	AAA	TAA	GTA	AAA	CCA	AAA	AAA	AAA	AAA	AA	

SUBSTITUTE SHEET

22/38

FIG. 19.

Nucleotide and deduced amino acid sequence for the male flower specific cDNA clone, pMS18.

			10				20				30				40			
ACA	GCA	GTA	GCA	AGA	GGG	ATA	GAG	CAA	GGC	CAC	ACA	CAC	ACA	CAC				
	50			60			70			80				90				
ACC	ACT	AGG	CTA	GGT	TAG	CCT	TTT	AAT	CGT	CGT	CGA	GAA	GCA	AGA				
			100			110			120			130						
AGG	GCG	CTG	CAC	CAA	GCA	GGC	AAG	CAA	GAA	GAG	AGC	CGA	TCG	ACC				
	140			150			160			170				180				
GAG	AGC	TAG	CAC	GCG	ATG	GCG	AGG	TCT	TGC	CAA	GAT	GAT	GGT	GGC				
					Met	Ala	Arg	Ser	Cys	Gln	Asp	Asp	Gly	Gly				
			190			200			210			220						
GCA	CGT	CTG	CTG	GCC	TTG	CGC	TGG	CGT	GTC	GAC	CGC	CGA	GGC	AGG				
Ala	Arg	Leu	Leu	Ala	Leu	Arg	Trp	Arg	Val	Asp	Arg	Arg	Gly	Arg				
	230			240			250			260				270				
AAC	ATC	AAG	ACC	ACG	ACG	ACG	GAG	AAG	AAG	GAC	GAC	GCG	GTG	GTG				
Asn	Ile	Lys	Thr	Thr	Thr	Thr	Glu	Lys	Lys	Asp	Asp	Ala	Val	Val				
			280			290			300			310						
CAG	CCG	CAG	AGG	TTC	CGC	CCT	TCG	ACC	GCC	TCG	GCG	CGG	CGC	GTC				
Gln	Pro	Gln	Arg	Phe	Arg	Pro	Ser	Thr	Ala	Ser	Ala	Arg	Arg	Val				
	320			330			340			350				360				
CCC	GGC	GTT	CGG	CGG	CCT	CCC	CGG	CGG	CAC	GAT	TCC	TGG	CAG	CAG				
Pro	Gly	Val	Arg	Arg	Pro	Pro	Arg	Arg	His	Asp	Ser	Trp	Gln	Gln				
			370			380			390			400						
CAT	TCC	CGG	GTT	CAG	CAT	GCC	CGG	CAG	CGG	CAG	CAG	CCT	ACC	CGG				
His	Ser	Arg	Val	Gln	His	Ala	Arg	Gln	Arg	Gln	Gln	Pro	Thr	Arg				
	410			420			430			440				450				
GTT	CAG	CTT	GCC	CGG	CAG	CGG	CAC	GAT	GCC	CCT	CTT	CGG	CGG	CGG				
Val	Gln	Leu	Ala	Arg	Gln	Arg	His	Asp	Ala	Pro	Leu	Arg	Arg	Arg				
			460			470			480			490						
CTC	CCC	GGG	CTT	CAG	CGG	CTT	CGG	CGG	CAT	GCC	CGG	GTC	GCC	CAC				
Leu	Pro	Gly	Leu	Gln	Arg	Leu	Arg	Arg	His	Ala	Arg	Val	Ala	His				

SUBSTITUTE SHEET

23/38

FIG. 19.

(cont.)

500	510	520	530	540
CGC CGG CTC CGT CCC CGA GCA CGC CAA CAA GCC CTG AAC GCC AAC				
Arg Arg Leu Arg Pro Arg Ala Arg Gln Gln Ala Leu Asn Ala Asn				
550	560	570	580	
AAG CGT GGT AGT AGA GGT GCT ACT GTT ACT GTA GTA CGT CGT CGT				
Lys Arg Gly Ser Arg Gly Ala Thr Val Thr Val Val Arg Arg Arg				
590	600	610	620	630
CTT CAT GCA TGC GTG GTT CGT GGT TTC CCT AGC TCC ATA CGA GCA				
Leu His Ala Cys Val Val Arg Gly Phe Pro Ser Ser Ile Arg Ala				
640	650	660	670	
GTA GTT GGG CTT GCA CGT ACC GTA CGT CTA GCT AGC TAT ATA TAT				
Val Val Gly Leu Ala Arg Thr Val Arg Leu Ala Ser Tyr Ile Tyr				
680	690	700	710	720
GCT TGT GTT CTA CTG CTT TTT AGT TTA ATT ACC TGC CTG CAT TGG				
Ala Cys Val Leu Leu Leu Phe Ser Leu Ile Thr Cys Leu His Trp				
730	740	750	760	
AGA GTT GGA TCT GTT TCA TTT GGT GGT GTT TGC TTT ACT ATT AGG				
Arg Val Gly Ser Val Ser Phe Gly Gly Val Cys Phe Thr Ile Arg				
770	780	790	800	810
TCA GTA TCT GTT TGT GGA GAC TTG GTG TTT AAT TTA TTT AGC CGT				
Ser Val Ser Val Cys Gly Asp Leu Val Phe Asn Leu Phe Ser Arg				
820	830	840	850	
TTG TGA CTG GTT GTA GCT AGC GGT GGT GCG GTG GTG ATG TTC TTG				
Leu				
860	870	880	890	900
AGG CAT GAA TAA TGC TAC ATG CAT GTG ATG TAT CCA TGT TTT GTG				
910	920	930		
TGT GGT AAA CCT GTT GTT TGT ATA AGC TGT CCC				

SUBSTITUTE SHEET

24/38

Restriction map of 9 kb EcoRI fragment from clone 10/CT8-3

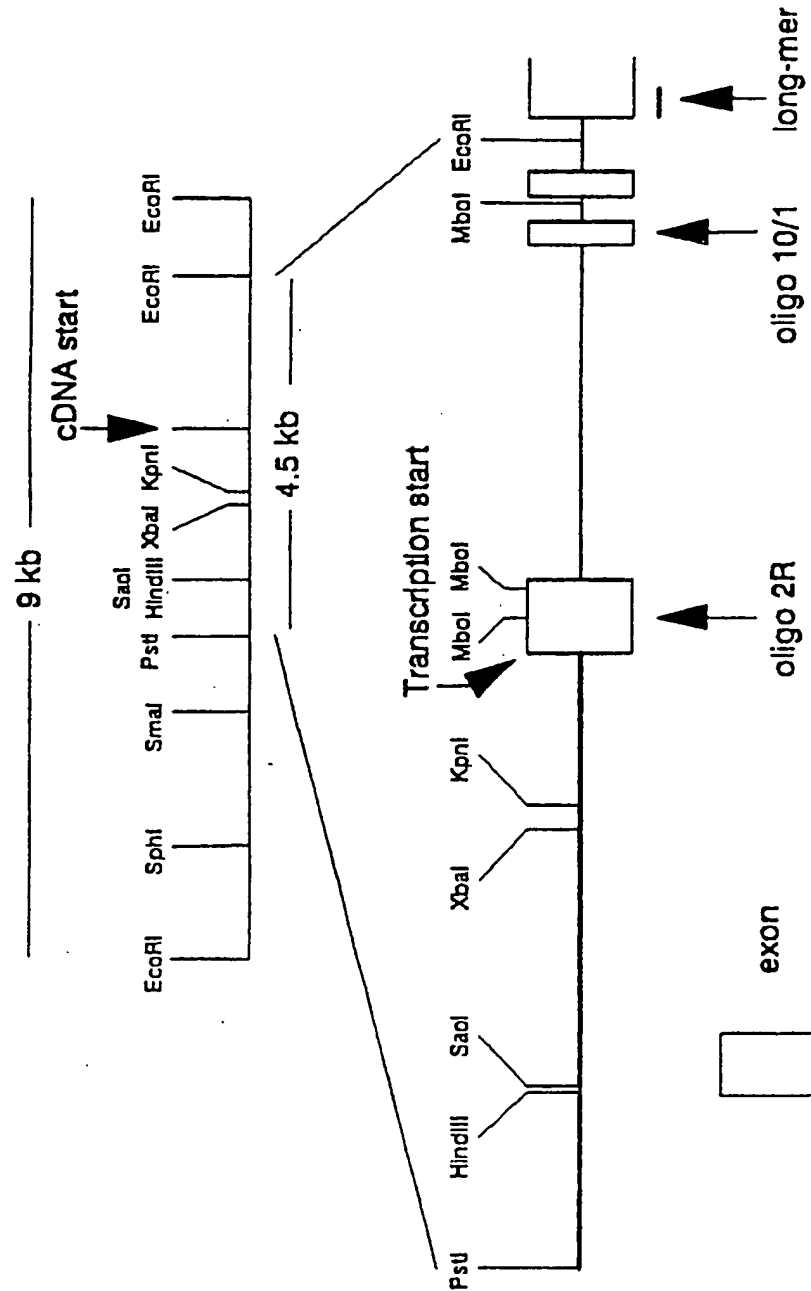


FIG. 20.

25/
38

Restriction map of 17 kb EcoRI fragment from clone 14/17M

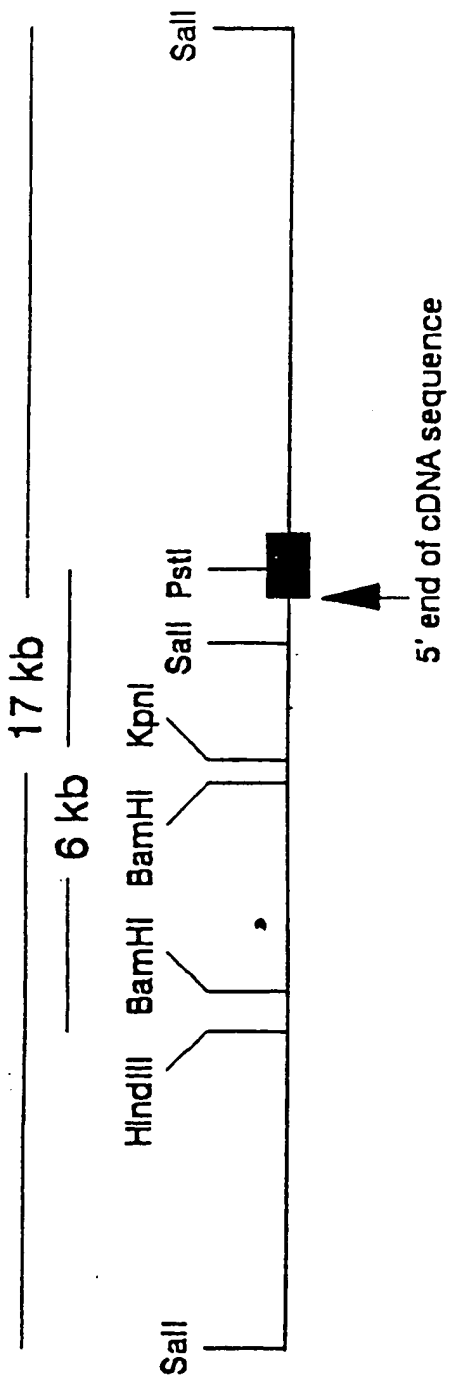


FIG. 21.

26/38

Restriction map of 16 kb EcoRI fragment from clone 18/CT3

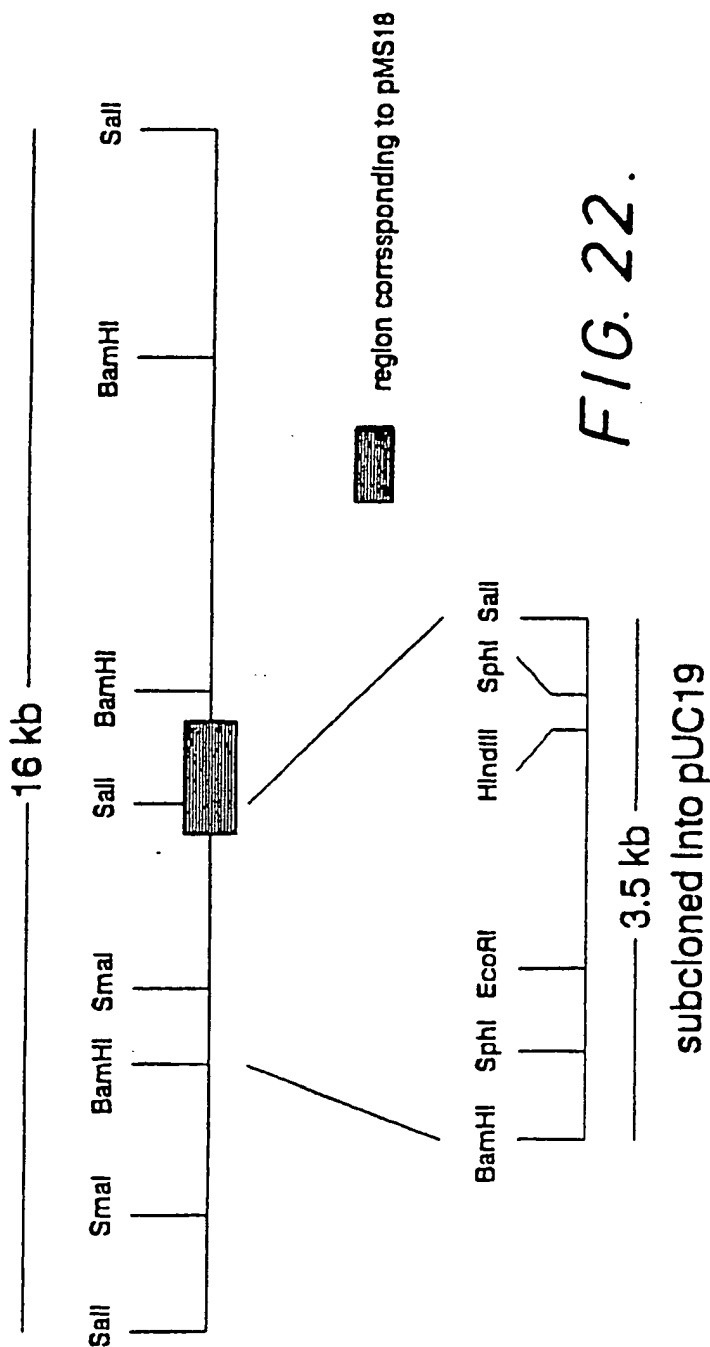


FIG. 22.

27/38

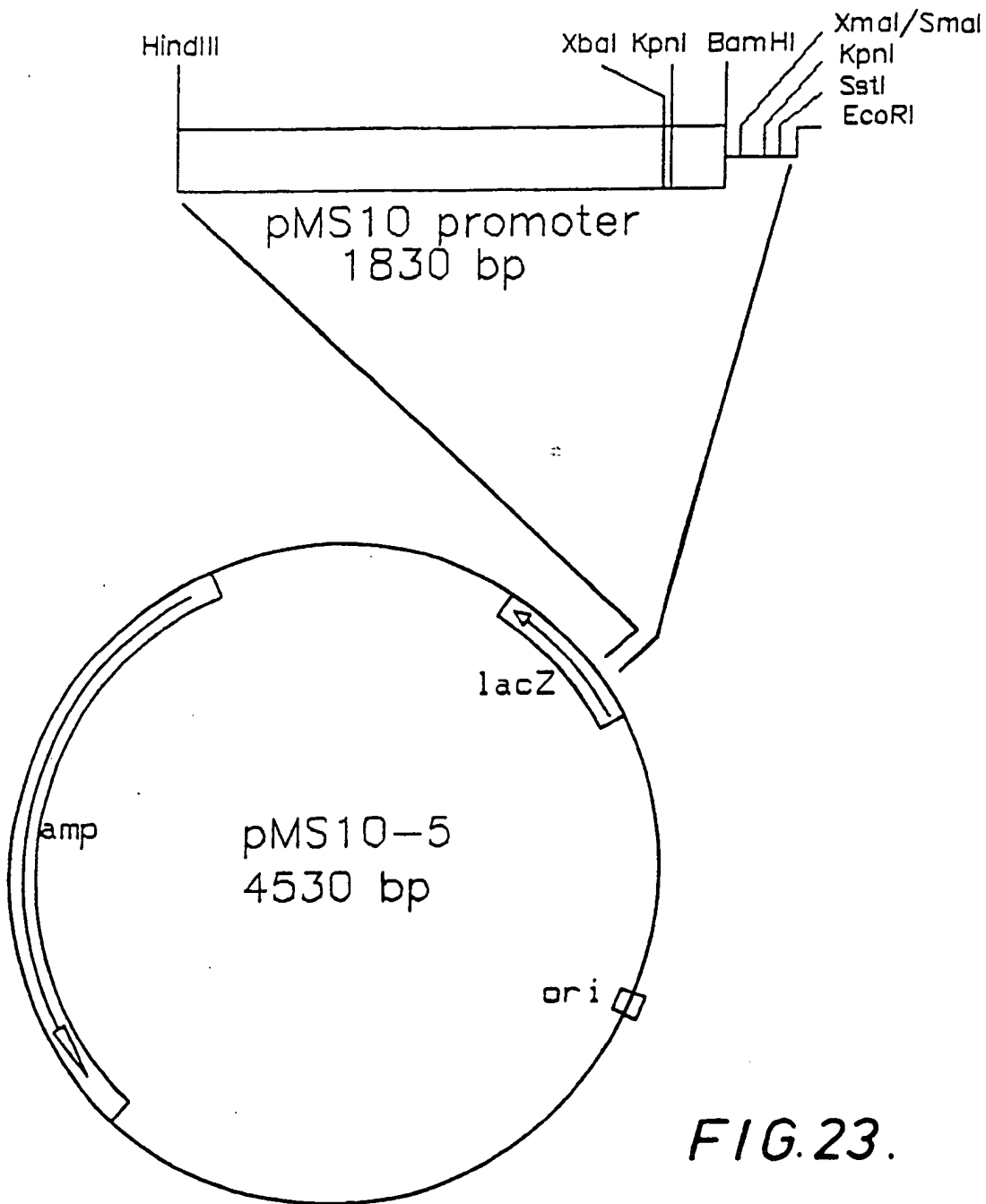


FIG. 23.

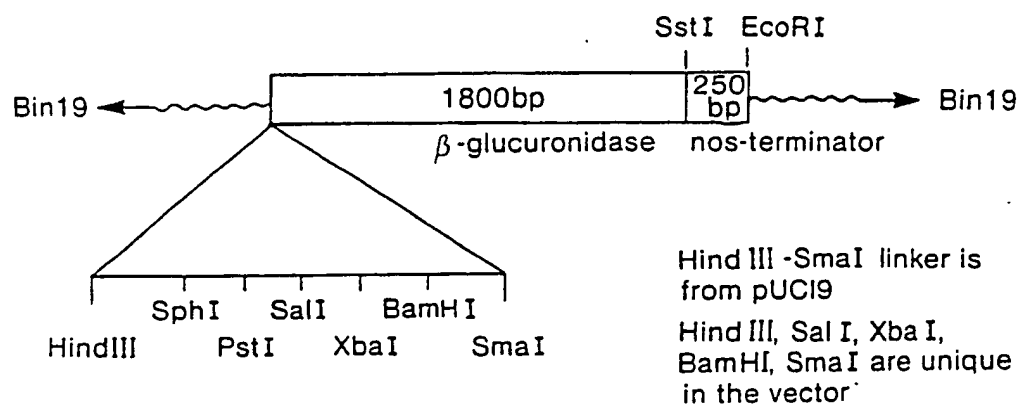
Clone pMS10-5.

SUBSTITUTE SHEET

28/38

FIG. 24.

Structure of pTAK1, pTAK2, pTAK3



pTAK1 GGATCCCCG GGT GGTCAGTCCCTT ATG
BamHI SmaI

pTAK2 GGATCCCCGG GTA GGTCAGTCCCTT ATG
SmaI

pTAK3 GGATCCCCGGG TAC GGTCAGTCCCTT ATG
SmaI

SUBSTITUTE SHEET

29/38

Map of clone pMS10-6GUS

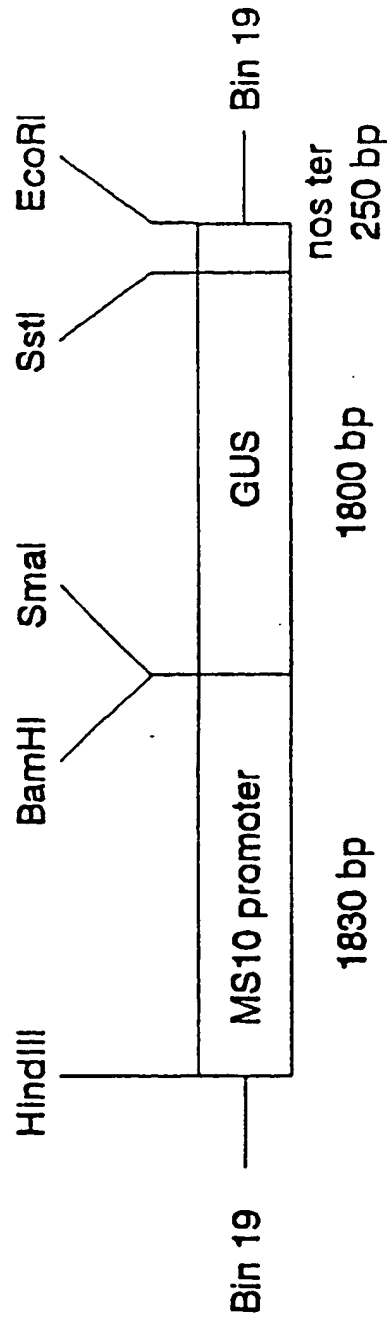


FIG. 25.

30/38

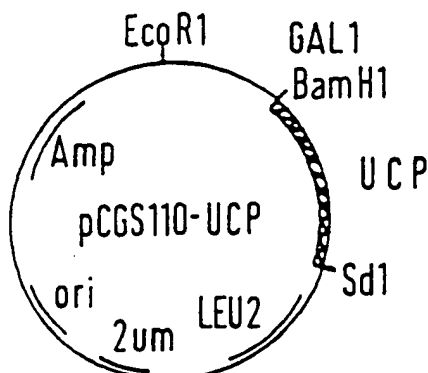


FIG. 26.

FIG. 27.

KpnI

```

1  UCGAAGUUGA GAGUUCGUA CCCACAUCAG GCAACAGUC CACUGUGUC
51  UUCAGGGCUG AUUCUUUUG GUCUCUGCCC UCCGAGCAA GAUGGUGAGU
101 UCGACAACU CCAGAGUGCA ACCCACCAG 666GUAAGA UCUCUCAGC
151 C66CGUUUCU GCCUGCUAG CAGACAUCAU CACCUUCCC CUGGACACC6
201 CCAAGUCC6 CCUCAGAU CAGGUGAAG GCCAGGCU CAGUACUUAU
251 AGGUUAAAAG GUGUCUAGG GACCAUCACC ACCUGGCCA AGACAGAAG6
301 AUUGCCGAAA CUGUACAGC GUCUGCCUGC UG6CAUCCAG AG6CAAUCA
351 GCUUUGCUUC CCUCAGGAU GGCUCUAC6 AUACGCUCA AGAGUACUUC
401 UCUCAG66A GAGAACGCC UGCCUCUUUG 66AAGCAAG UCUC66CUG6
451 CUUGAUGAG6 66UGCGUG6 C66UAUUCAU U66CAGCCC ACAGAGGUG6
501 UGAAGGUCAG AAGCAAGCA CAAAGCCAUC UGCAGG6AU CAAACCC6C
551 UACAGUG6A CCUACAAUGC UACAGAGUU AUAGCCACCA CAGAA6CUU
601 GUCAACACUG UGAAAGGGA C6ACUCCUAA UCUAAGAGA AUUGUCAUCA
651 UCAACUGUAC AGAGCUGUG ACAUAUGACC UCAUGAAG66 66CCCUUGUG
701 AACCACCACA UACUG6CAG UGAGGUC6CC UGCCAUUAC UGUCAGCUCU
751 UGUC6CG66 UUU6CAGCA CACUCCUG6C CUCUG66UG GAUGG6UAA
801 AAAGAGAUU CAUACUCUCU CUACAGGAC AGUACCCAAG UGUACCCAGC
851 UGUGCAAUGA CCAUGUACAC CAAGGAAGGA C66CAGCCU UUUCAAAG6
901 GUUUGCGCCU UCUUUCUGC GACUGG6AUC CUGGAGCUC AUCAUGUUG
951 UGUGCUUGA ACAGCUGAG AAAGAGCUGA UGAAGUCC66 GCAGACAGUG
1001 GACUGCAGCA CAAGG6CAG UUGGAGAAAG 66AUGCUAAA CACCAUG66
1051 CUCCUAGCU 66CUCCUUAU GCUGGAGAG CAGAAUAAA ACCAACCAA
1101 GAAUCAGAC G

```

SUBSTITUTE SHEET

3/38

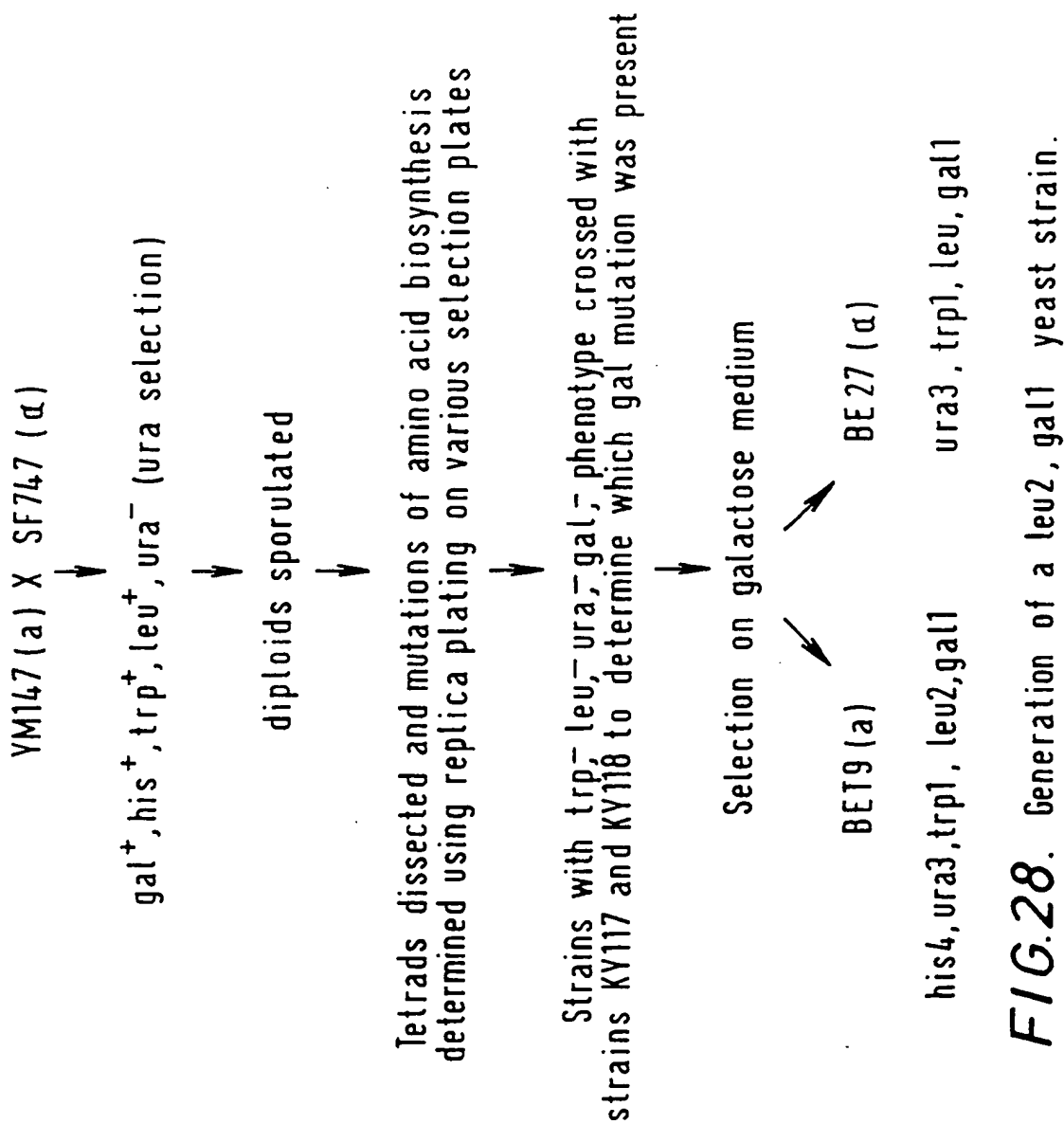


FIG.28. Generation of a $leu2, gal1$ yeast strain.

SUBSTITUTE SHEET

32/38

	BET9	BET9 PKV	BET9 UCP	BET9	BET9 PKV	BET9 UCP
YPD	***	***	***	***	***	***
YPD+Gal	***	***	***	***	***	***
YPDG	***	***	***	***	***	***
YPDG+Gal	***	***	***	***	***	***
MMGlu	***	***	***	***	***	***
MMGlu+Gal	***	***	***	***	***	***
MMLac	***	***	***	***	***	***
MMLac+Gal	***	***	***	***	***	***
MMGly	***	***	***	***	***	***
MMGly+Gal	***	***	***	***	***	***
LacCas	***	***	***	***	***	***
LacCas+Gal	***	***	***	***	***	***
GlyCas	***	***	***	***	***	***
GlyCasGal	***	***	***	***	***	***
2%Lac	***	***	***	***	***	***
2%Lac+Gal	***	***	***	***	***	***

FIG. 29.

SUBSTITUTE SHEET

33/38

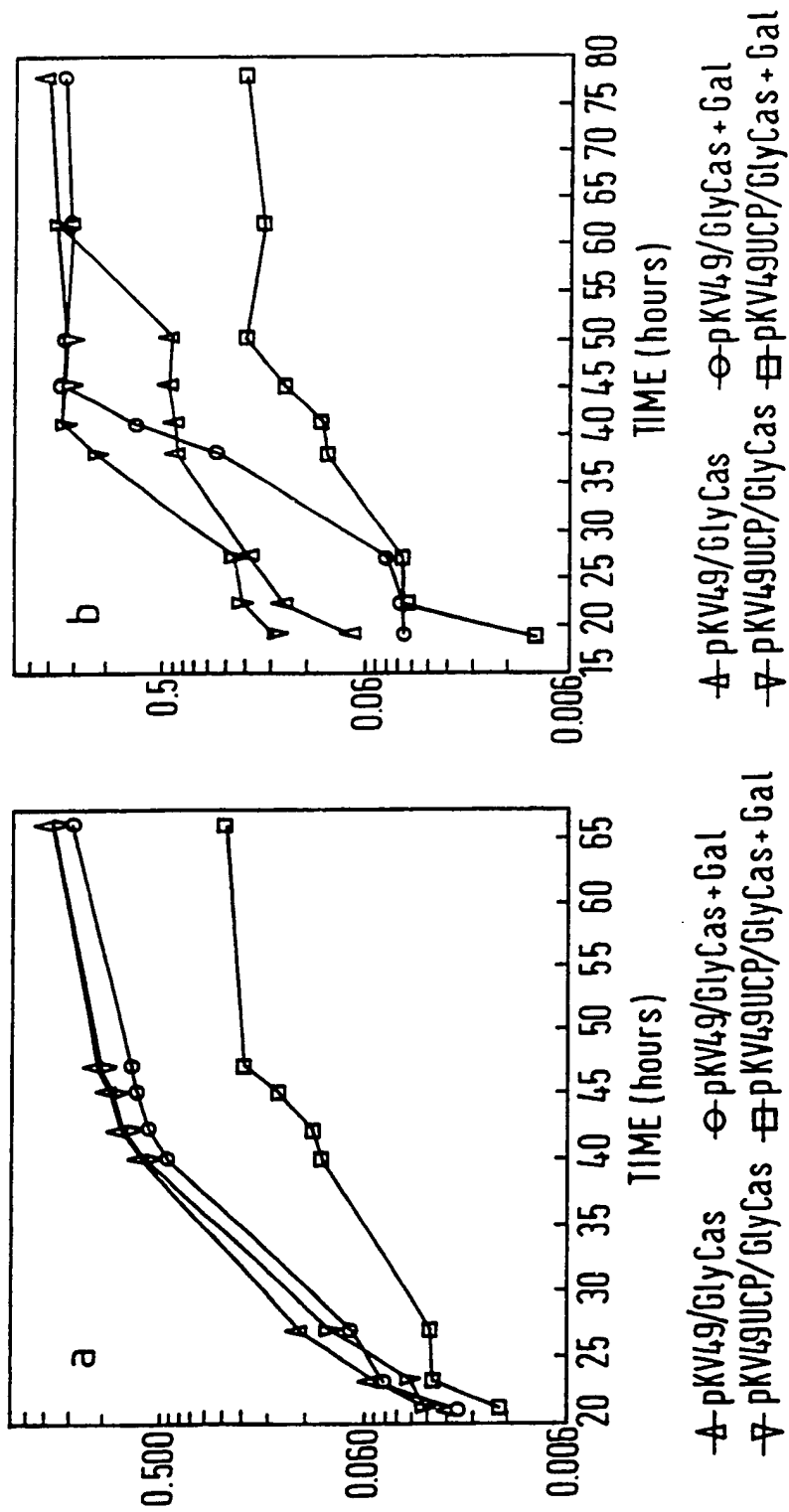


FIG. 30.

SUBSTITUTE SHEET

34/38

FIG. 31.

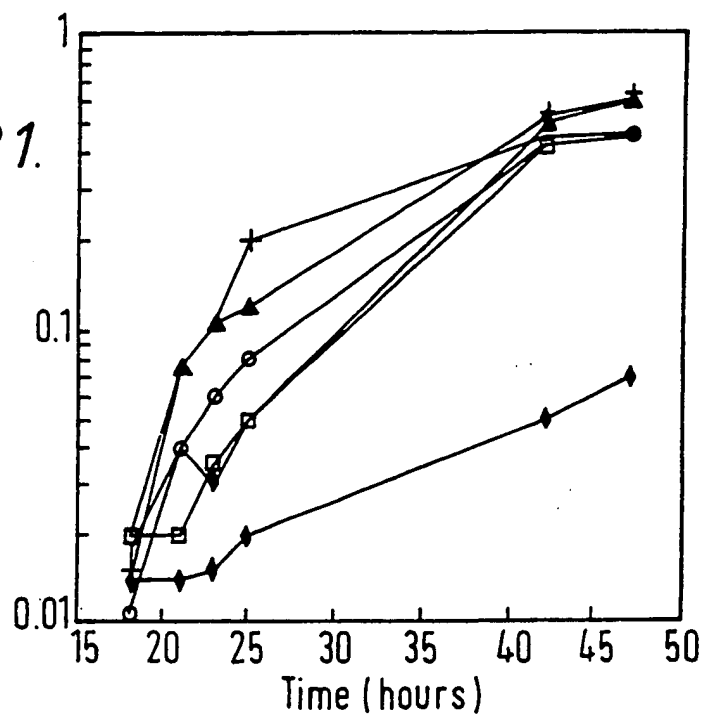
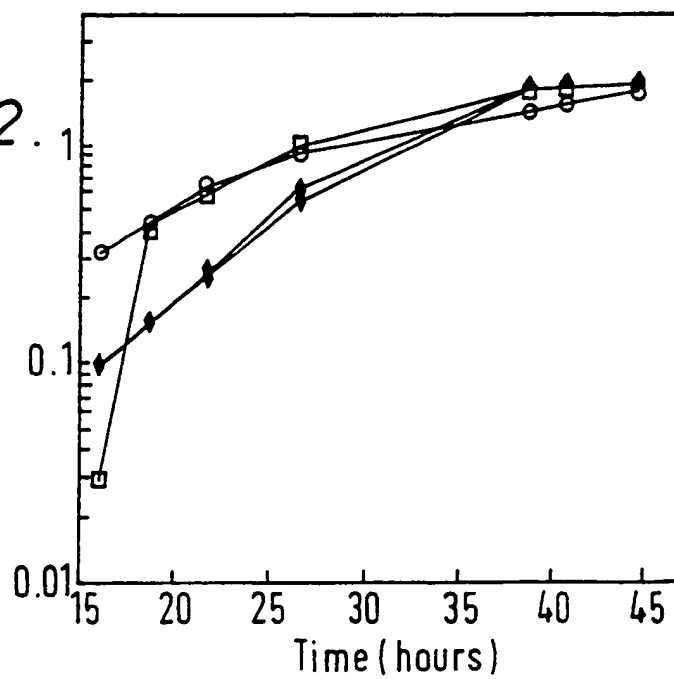


FIG. 32.



SUBSTITUTE SHEET

35/38

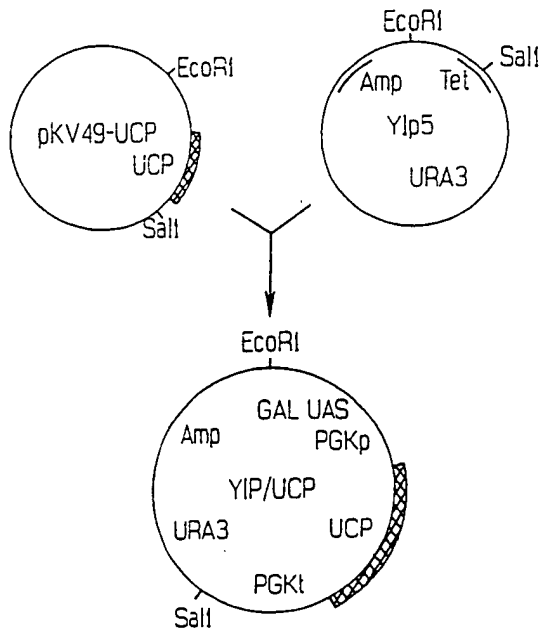


FIG. 33.

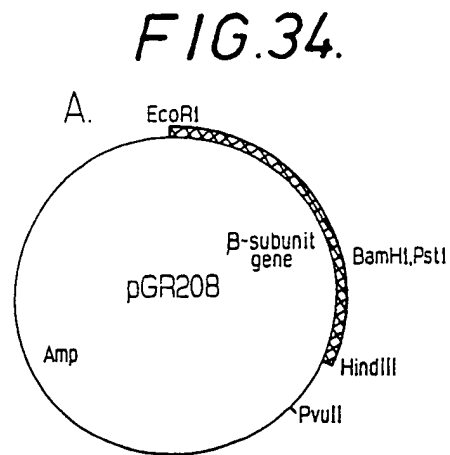


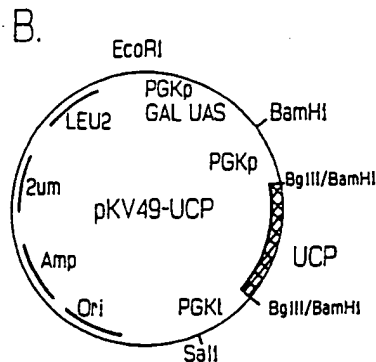
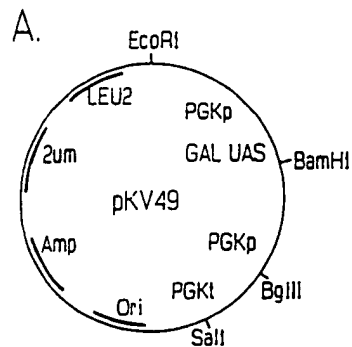
FIG. 34.

B.

Oligo B1 w.t. ACGGTCAAATGAACGAACCTC
mut. ACGGTCAAATAACGAACCTC

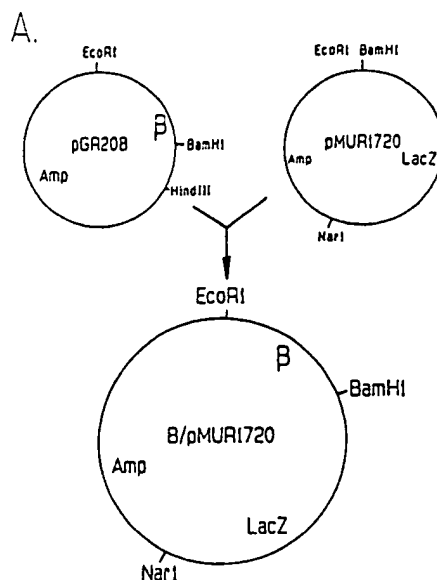
Oligo B2 w.t. AGGTGTCGGTAAGACTGTGTT
mut. AGGTGTCGGTCAGACTGTGTT

FIG. 35.



SUBSTITUTE SHEET

36/38



B.

Fusion Junction

ATT TAC CCT GCA GTG GAT CCC GTC GTT TTA

Ile Tyr Pro Ala Val Asp Pro Val Val Leu

345 346 347 348 349 350 9 10 11

beto - subunit Loc - Z

FIG. 36. Construction of β -subunit / β -galactosidase fusion protein

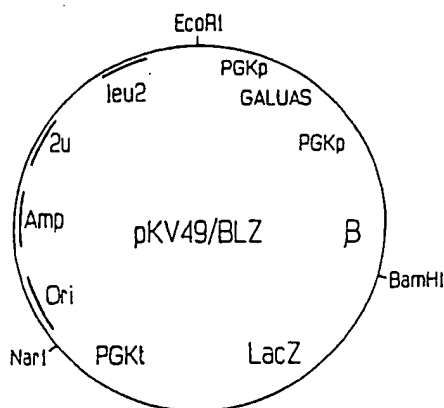
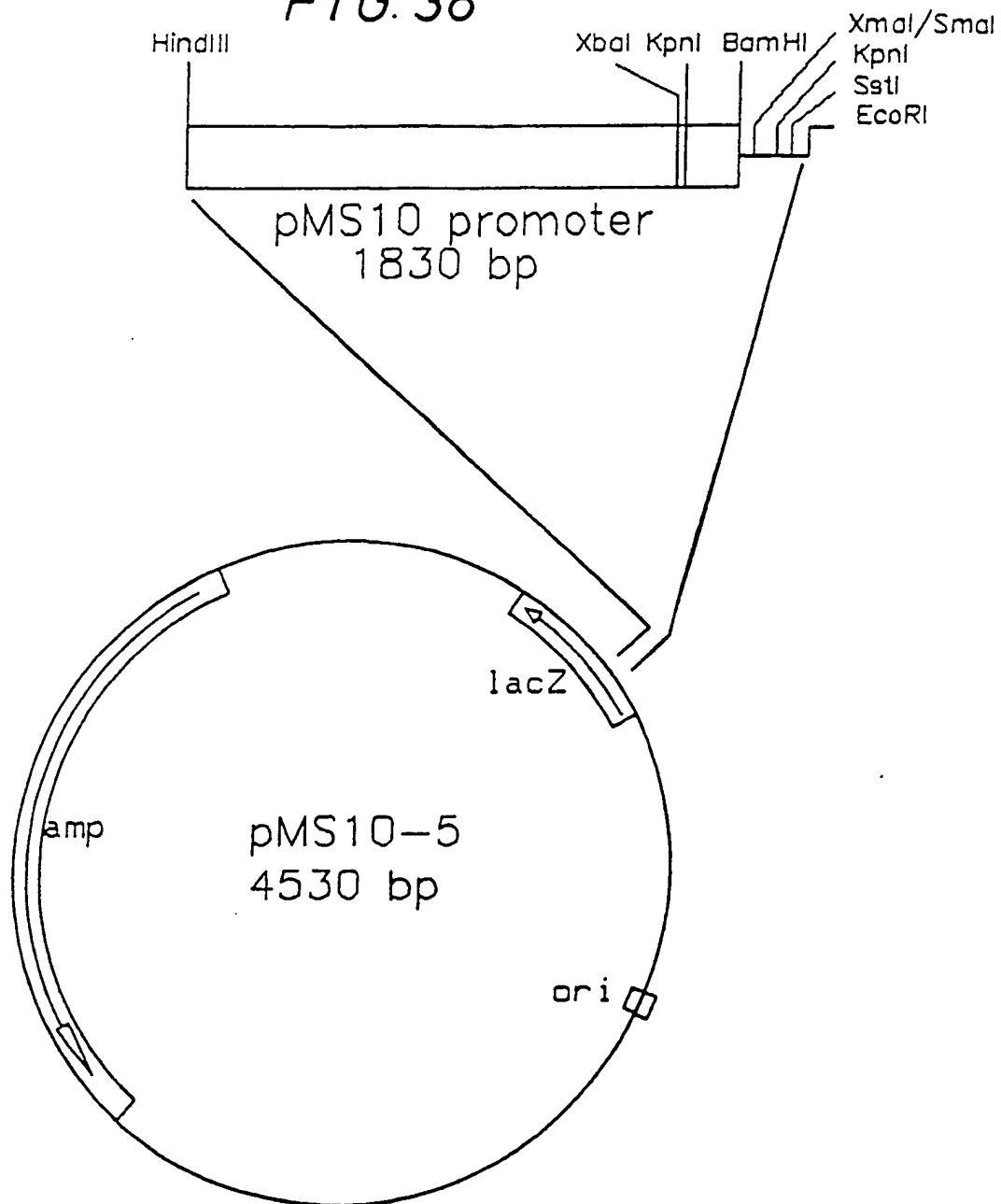


FIG. 37. Plasmid map of the construct pKV49/BLZ

37/38

FIG. 38



SUBSTITUTE SHEET

38/38

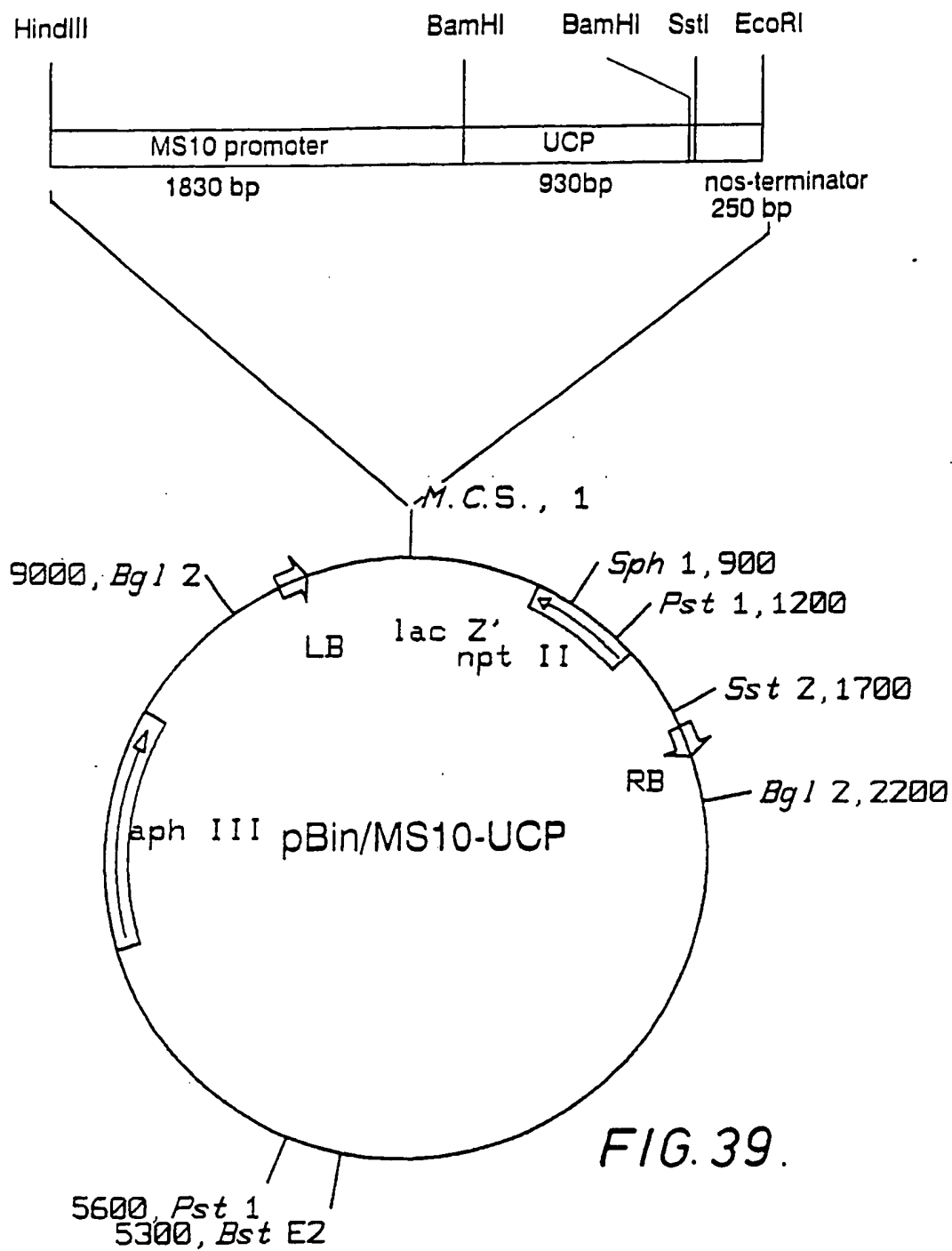


FIG. 39.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 90/00110

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ¹		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 12 N 15/82, 5/10, A 01 H 5/00, // C 12 N 15/54, 15/12, 15/55, 15/29		
II. FIELDS SEARCHED		
Minimum Documentation Searched ²		
Classification System ¹	Classification Symbols	
IPC ⁵	C 12 N, A 01 H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ³		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁴		
Category ⁵	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	EP, A, 0329308 (PALADIN HYBRIDS) 23 August 1989 see page 6, lines 14-45	1
P,A	--	2-30
P,X	EP, A, 0332104 (CIBA-GEIGY) 13 September 1989 see page 17, lines 34-40	1
P,A	--	2-30
P,A	WO, A, 89/10396 (PLANT GENETIC SYSTEMS) 2 November 1989 see the whole document	1-30

<p>* Special categories of cited documents: ¹⁴</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"G" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
8th May 1990	- 5. 06. 90	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	MISS T. LAZELAAR	

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB 9000110

SA 33737

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/05/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0329308	23-08-89	AU-A- 2963289	03-08-89
EP-A- 0332104	13-09-89	AU-A- 3108089	14-09-89
WO-A- 8910396	02-11-89	AU-A- 3537189	24-11-89
		EP-A- 0344029	29-11-89

EPO FORM P0019

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82